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13. ABSTRACT (Maximum 200 Words)

The overall goal of these studies was to characterize the regulation and expression of C/EBPδ in finite-lifespan and immortalized human mammary epithelial cells (HMECs), human breast cancer cell lines and human breast cancer samples. We demonstrate the induction of C/EBPδ mRNA and protein and G_0 growth arrest of finite-lifespan HMECs and immortalized mammary epithelial cells (MCF-10A, MCF-12A) following serum and growth factor withdrawal and IL-6 family cytokine addition. Induction of C/EBPδ is reduced in human breast cancer cell lines (MCF-7, MDA-MB-231, T-47D, SK-BR-3, SUM-44PE) in response to serum and growth factor withdrawal, and only 2/7 growth arrest. Addition of IL-6 family cytokines increased C/EBPδ mRNA and protein levels but only 3/7 growth arrested. C/EBPδ is constitutively expressed in the SUM-102PT cell line and is not expressed in the SUM-52PE cell line. Human breast cancer samples also exhibit decreased C/EBPδ expression. Analysis for base sequence changes in the C/EBPδ gene revealed a nonsense mutation (A538T) in the SUM-52PE cell line and 3 silent mutations in breast cancer cell lines and 97 human primary breast cancer samples. Limited methylation was identified in the T-47D cell line and in 6/17 primary breast tumors that were selected for low C/EBPδ expression.

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INTRODUCTION

Inactivation of tumor suppressor genes, through a variety of mechanisms, is a key pathological step in breast cancer development and progression. The overall goal of this research is to investigate the regulation, expression and clinical relevance of CCAAT/Enhancer binding protein δ (C/EBPδ, CEBPD) as a candidate tumor suppressor gene in breast cancer. Our hypothesis is that C/EBPδ is a key regulator of mammary epithelial cell growth arrest and that loss in C/EBPδ function is part of the cascade of molecular events that facilitate the progression to metastatic breast cancer. In previous reports we have shown that C/EBPδ expression is associated with growth arrest in mouse mammary epithelial cells. The data presented in this research report demonstrate the association between STAT3 activation, C/EBPδ gene expression and growth arrest in human mammary epithelial cells. "Loss of function" alterations in the Stat3/C/EBPδ are common in human breast cancer cell lines. In addition, we show that the C/EBPδ gene exhibits relatively few nucleotide sequence alterations in clinical breast cancer. The C/EBPδ gene promoter, however, exhibits extensive CpG methylation and gene silencing in the SUM-52PE cell line and variable CpG methylation that is associated with reduced C/EBPδ gene expression in primary human breast cancer samples. Hence, alterations in the Stat3/C/EBPδ growth inhibitory pathway may play an important role in the etiology or progression of breast cancer.

BODY

1. Objective #1: Investigate C/EBPδ gene expression during the induction of G_0 growth arrest in human mammary epithelial cell lines.

- ***Develop an optimized cell culture system for each human mammary-derived cell line to exit the cell cycle and enter G_0 growth arrest.*** We developed an optimized cell culture system for primary, finite-lifespan human mammary epithelial cells (Clonetics), immortalized, non-tumorigenic human mammary epithelial cells (MCF-10A, MCF-12A) and human breast cancer cell lines (MCF-7, SK-BR-3, T-47D, MDA-MB-231, SUM-44PE, SUM-52PE, SUM-102PT). Cells were growth arrested by serum and growth factor withdrawal and by the addition of IL-6 family cytokines (IL-6, Oncostatin M).
- ***Document G_0 growth arrest by flow, northern and western blotting (all methods in place).*** We documented the ability or inability of the cells to growth arrest by Northern blotting, Western blotting, ^{3}[H]thymidine incorporation analysis, and flow cytometry.
- ***Complete G_0 growth arrest analysis of all cell lines, carry out statistical analysis of data.*** We have consistently observed that growth arrest treatments – contact inhibition, serum and growth factor withdrawal and IL-6 family cytokines increase Stat3 phosphorylation, increase C/EBPδ gene expression and induce growth arrest of finite-lifespan (Clonetics) and immortalized but non-transformed human mammary epithelial cell lines (MCF-10A, MCF-12A). In contrast, we have observed variable C/EBPδ gene expression and growth arrest in human breast cancer cell lines (MCF-7, SK-BR-3, T-47D, MDA-MB-231, SUM-44PE). We have also identified two human breast cancer cell lines that exhibit novel C/EBPδ gene

expression patterns. SUM-102PT cells express elevated levels of C/EBPδ regardless of growth conditions and do not growth-arrest under standard growth arrest conditions. These results suggest that the SUM-102PT cell line is insensitive to growth arrest regulated by C/EBPδ. In contrast, SUM-52PE cells do not express C/EBPδ under any condition that we have been able to test. We statistically analyzed growth arrest data by one-way analysis of variance (ANOVA). Results of all cell lines are included in the attached paper submitted for publication in Experimental Cell Research. Further analysis of the SUM-52PE cell line is discussed in Objectives #2 and #3 below.

2. Objective #2: Investigate C/EBPδ gene expression and promoter methylation status in breast cancer cell lines and human breast cancer and noninvolved breast tissue samples.

- ***Complete archive of human breast cancer tissue samples for clinical status, steroid hormone status, presence of metastasis and availability of noninvolved tissue.*** We have completed archival of clinical status information of tissue samples obtained from the CHTN to be used in C/EBPδ gene expression studies (Objectives #2, #3).
- ***Optimize RT-PCR method with both C/EBPδ and beta-actin primer pairs using total RNA from human cell lines and clinical samples.*** PCR optimization was completed; however, C/EBPδ gene expression levels are adequate for analysis by Northern blot.
- ***Isolate high molecular weight DNA from human cell lines and clinical samples, carry out restriction digest with methylation sensitive isoschizomers and southern blot, probe with C/EBPδ 5' end probe.*** High molecular weight DNA was successfully isolated from human cell lines and clinical samples and was restriction digested with methylation sensitive isoschizomers. The results indicate that the C/EBPδ gene promoter has multiple small molecular weight bands that are released following digestion with 4 base pair cutter methylation sensitive restriction enzymes (*HpaII, MspI*). Since this experimental approach did not provide the best method to assess promoter methylation status, we utilized the more sensitive bisulfite treatment followed by direct genomic sequencing or methylation-specific PCR.
- ***Develop primers and implement bisulfite genomic sequencing method.*** Primers for amplification of the bisulfite-treated genomic DNA have been designed and genomic sequencing was performed by the OSU Neurobiotechnology Sequencing Center.
- ***Complete analysis of human cell lines and clinical samples for each of the above methods, carry out statistical analysis of data.*** We have completed analysis of C/EBPδ gene expression in human breast cancer cell lines and breast cancer primary samples. We have performed methylation status analysis on breast cancer cell lines and primary breast cancer samples. We previously demonstrated that the SUM-52PE cell line does not express C/EBPδ mRNA or protein under growth arrest conditions (Objective #1). We now show that the C/EBPδ gene promoter is heavily methylated (26/27 CpGs) in the SUM-52PE cell line. We also show variable methylation of the C/EBPδ gene promoter in the T-47D breast cancer cell line and in low C/EBPδ gene expressing primary breast cancer samples. The abstract describing these results was presented at the US Army Era of Hope Meeting, Orlando, FL, 9/25-9/29/02 (see Reportable Outcomes).

3. Objective #3: Investigate the type, frequency and distribution of somatic alterations in the C/EBPδ gene in breast cancer cell lines and primary breast cancer samples.

- ***Develop and optimize primers for amplification of genomic DNA from human cell lines and clinical samples for cold SSCP analysis.*** All primers have been designed and tested for functional utility in human breast cancer cell lines and primary breast cancer samples.
- ***Optimize gel conditions for detection of cold SSCP products.*** Gel conditions (temperature, gel buffers, acrylamide concentration, time, etc.) have been optimized to detect SSCP bands.
- ***Complete cold SSCP analysis of human cell lines and clinical samples. Sequence suspected mutants, identify altered bases, compare with normal samples.*** Cold SSCP analysis of human breast cancer cell lines and primary breast cancer samples has been completed. The results have been published in Molecular and Cellular Probes (see Reportable Outcomes).

KEY RESEARCH ACCOMPLISHMENTS

- The direct association between C/EBPδ gene expression and G_0 growth arrest in mammary epithelial cells has been extended to finite-lifespan and non-transformed immortalized human mammary epithelial cells. These results are consistent with our previous data in mouse cell lines and in the C/EBPδ knockout mouse. It indicates that C/EBPδ plays a pivotal role in mammary epithelial cell growth arrest.

- The direct association between C/EBPδ gene expression and growth arrest is disrupted in human breast cancer cell lines. In SUM-52PE cells, the C/EBPδ gene is silenced.

- C/EBPδ gene promoter methylation analysis using bisulfite/genomic sequencing demonstrated that the SUM-52PE C/EBPδ gene promoter is heavily methylated. Our analysis indicated that 26/27 CpGs in the SUM-52PE C/EBPδ gene promoter were methylated. C/EBPδ gene promoter methylation was also demonstrated in breast cancer samples. The typical C/EBPδ gene promoter methylation in clinical breast cancer samples was less dense than the C/EBPδ gene promoter in the SUM-52PE cell line. We propose to further investigate the significance of C/EBPδ gene promoter methylation in C/EBPδ gene silencing in primary breast cancer in future studies.

- We have analyzed the base sequence changes (polymorphisms) in the C/EBPδ gene in 5 human breast cancer cell lines and 97 human breast cancer samples. We have established that the frequency of base sequence alterations in the C/EBPδ gene is relatively low. These data have been published in Molecular and Cellular Probes.

REPORTABLE OUTCOMES

- **Manuscript published:** D. Tang, J. DeWille, Detection of base sequence changes in the CEBPD gene in human breast cancer cell lines and primary breast cancer isolates, Mol.Cell Probes 17 (2003) 11-14.

- **Manuscript submitted to Experimental Cell Research:** G. Sivko, J. DeWille, Progressive "Loss of Function" Alterations in the STAT3/C/EBPδ Growth Control Pathway: Comparative Analysis of Primary and Immortalized Human Mammary Epithelial Cells and Human Breast Cancer Cell Lines.

- **Poster presentation:** The CCAAT/Enhancer Binding Protein δ (C/EBPδ) Gene Promoter is Induced by Activated Signal Transduction and Activator of Transcription 3 (STAT3) and Silenced by Hypermethylation. Tang, D., Sivko, G.S., Zhang, Y., and DeWille, J.W. Presented at The Ohio State University Comprehensive Cancer Center Fifth Annual Scientific Meeting (Abstract #99), April 18, 2003, Columbus, OH.

- **Poster presentation:** The CCAAT/Enhancer Binding Protein δ (C/EBPδ) Gene Promoter is Induced by Activated Signal Transduction and Activator of Transcription 3 (STAT3) and Silenced by Hypermethylation. Tang, D., Sivko, G.S., Zhang, Y., and DeWille, J.W. Presented at American Association of Cancer Research for 94th Annual Meeting (Abstract #4235), July 11-14, 2003, Washington, D.C.

- **Abstract presented:** "C/EBP-delta: a potential breast cancer tumor suppressor gene". Tang, D., Sivko, G and JW DeWille. Era of Hope Department of Defense Breast Cancer Research Program Meeting, September 25-29, 2002.

- **Poster presentation:** Increased CCAAT/Enhancer Binding Protein δ (C/EBPδ) Expression in Growth Arrested Human Breast Derived Cell Lines. G. S. Sivko and J. W. DeWille. 92nd Annual Meeting of American Association for Cancer Research, New Orleans, LA, March 24-28, 2001.

- **Poster presentation:** C/EBPδ Expression in Human Mammary Epithelial and Prostate Cells. Sivko, G., Sanford, D., and DeWille, J. The Ohio State University Comprehensive Cancer Center Third Annual Scientific Meeting, January 24, 2001, Dublin, OH, p.36

- **Presentation:** CCAAT/Enhancer Binding Protein δ (C/EBPδ): A Candidate Breast Cancer Tumor Suppressor Gene. Sivko, G. and J. DeWille. 12th Annual Molecular Biology and Cancer Genetics Program Stone Lab Meeting, The Ohio State University Comprehensive Cancer Center, September 15-17, 2000.

CONCLUSIONS

The overall goal of this research is to investigate the structure and expression of the C/EBPδ gene in human breast cancer cell lines and primary breast cancer samples. The research described in this final report indicates that C/EBPδ gene expression is tightly linked to growth arrest in normal (finite-lifespan) and non-transformed immortalized human mammary epithelial cells. A more variable picture emerged from the breast cancer cell lines, indicating that growth arrest involving C/EBPδ gene expression is probably compromised in breast cancer cells. To investigate the mechanism by which C/EBPδ gene expression is altered in human breast cancer, we sequenced the C/EBPδ gene in genomic isolates from breast cancer cell lines and primary breast cancer samples. The results revealed few base alterations. We next investigated the promoter methylation status and found that the C/EBPδ gene promoter is heavily methylated in the SUM-52PE cell line, a cell line that does not express the C/EBPδ gene. We also identified "strategically" methylated bases in the C/EBPδ gene promoter in the T-47D cell line and in primary breast cancer isolates.

To better address the problem, we have extended our studies beyond the funded objectives of this grant and performed in situ hybridization on clinical breast cancer sections. These preliminary results indicate that C/EBPδ gene expression declines during malignancy. This is exactly what we would predict from the results that we have obtained in these and previous studies.

In the "so what section", we would respond that this work has identified a new tumor suppressor gene. Although mutational inactivation does not appear to be a major mechanism of C/EBPδ gene "loss of function" in breast cancer, promoter silencing by methylation has been demonstrated in these studies.

REFERENCES

All references are provided in combination with the attached manuscripts located in the appendix section of this report.

APPENDICES

I. **Journal Article Reprint:** D. Tang, J. DeWille, Detection of base sequence changes in the CEBPD gene in human breast cancer cell lines and primary breast cancer isolates, Mol.Cell Probes 17 (2003) 11-14.

II. **Manuscript Submitted:** G. Sivko, J. DeWille, Progressive "Loss of Function" Alterations in the STAT3/C/EBPδ Growth Control Pathway: Comparative Analysis of Primary and Immortalized Human Mammary Epithelial Cells and Human Breast Cancer Cell Lines.

III. **Poster Presenting Methylation Data:** Tang, D., Sivko, G.S., Zhang, Y. and DeWille, J.W. American Association for Cancer Research 94th Annual Meeting (Abstract #4235).

IV. **Abstract from Poster Presentation:** Tang, D., Sivko, G.S., Zhang, Y. and DeWille, J.W. The Ohio State University Comprehensive Cancer Center Fifth Annual Scientific Meeting (Abstract #99).

V. **Abstract from Poster Presentation:** Tang, D., Sivko, G.S., Zhang, Y. and DeWille, J.W. American Association for Cancer Research 94th Annual Meeting (Abstract #4235).

VI. **Abstract Presented:** Tang, D., Sivko, G., DeWille, J.W. Era of Hope Department of Defense Breast Cancer Research Program Meeting.

VII. **Abstract from Poster Presentation:** Sivko, G.S., DeWille, J.W. American Association for Cancer Research 92nd Annual Meeting (Abstract #3962).

VIII. **Abstract from Poster Presentation:** Sivko, G., Sanford, D., DeWille, J. The Ohio State University Comprehensive Cancer Center Third Annual Scientific Meeting.

IX. **Abstract from Poster Presentation:** Sivko, G., DeWille, J. 12th Annual Molecular Biology and Cancer Genetics Program Stone Lab Meeting.

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Detection of base sequence changes in the CEBPD gene in human breast cancer cell lines and primary breast cancer isolates

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Abstract

Single strand conformation polymorphism (SSCP) analysis was performed on genomic DNA from 97 human breast cancer samples and 10 breast cell lines to screen for mutations in the single exon C/EBPD gene. Three C → T transitions resulting in silent mutations were detected in three individual breast cancer samples. One breast cancer sample also contained a G → T transversion (Q253H). The SUM-52PE cell line contained an A → T transversion ($\bar{A}AG \rightarrow \bar{T}AG$) resulting in a nonsense mutation (K180Stop). All mutations identified in genomic DNA isolates were in highly conserved regions of the C/EBPD gene. This study indicates that mutational alterations in the coding region of the C/EBPD gene are relatively uncommon in human breast cancer.

Keywords: C/EBPD; Single strand conformation polymorphism (SSCP); Genomic sequencing; Breast cancer

1. Introduction

CCAAT/enhancer binding proteins (C/EBPs) are members of the leucine zipper family of transcriptional factors [1]. C/EBPs play key roles in the control of cell growth and the expression of differentiation-specific genes [1,2]. Alterations in the structure and expression of C/EBP family members have been reported in a variety of human cancers and human cancer-derived cell lines. The first C/EBP family member directly linked to human cancer was C/EBPζ (C/EBP-Homologous Protein10 (CHOP10), GADD153). Virtually 100% of human mixoid liposarcomas contain a characteristic chromosomal translocation, (t(12;16)(q(13;p11) [3]. This translocation results in the formation of a TLS-CHOP fusion gene that is directly implicated in tumor pathogenesis [3]. Loss of function mutations in C/EBPα have been detected in 7% (10/150) of acute myeloid leukemia (AML) patients [4]. C/EBPα gene expression is markedly reduced in 43% (23/53) of primary lung cancer specimens [5]. Single nucleotide alterations resulting in missense mutations have been identified in the C/EBPβ gene in the Kc122 human chronic myelogenous leukemia cell line [6].

Previous studies from our laboratory have demonstrated that C/EBPδ plays a key role in the initiation and maintenance of mouse and human mammary epithelial cell G_0 growth arrest [7–11]. The growth arrest function of CEBPD (C/EBPδ) is important in human disease as a progressive decline in C/EBPD gene expression has been correlated with malignant breast cancer progression [12]. Recently, Angeloni et al. reported a silent mutation (C306A single nucleotide polymorphism) in the C/EBPδ gene in DNA isolates from normal healthy individuals and human lung cancer patients [13]. In this study, we investigated the presence of base alterations in the human C/EBPD gene in primary breast cancer isolates and breast cancer cell lines using the single strand conformation polymorphism (SSCP) assay and direct genomic DNA sequencing. The results indicate that base sequence changes in the C/EBPD gene are detectable at relatively low frequency in human breast cancer primary isolates.

2. Materials and methods

2.1. Samples preparation

Ninety-seven (97) clinical breast tissue samples were obtained from the Co-operative Human Tissue Network

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(CHTN), Tissue Procurement Division Ohio State University Comprehensive Cancer Center. SUM-52PE, SUM-44PE and SUM102PE cell lines were generously provided by Dr Stephen Ethier, Department of Radiation Oncology, University of Michigan. High molecular weight DNA was prepared from the clinical samples and cell lines as described previously [14]. Genomic DNA from MDA-MB-175, MDA-MB-231, SK-BR-3, BT-483, T-47D, ZR-75 and MCF-10A human breast cancer cell lines were purchased from ATCC (Manassas, VA).

2.2. Polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) assay

PCR-SSCP analysis of the single exon, 807 base-pair C/EBPδ coding region was performed as described by Orita et al. [15]. Four primer pairs were designed to provide overlapping amplified PCR products for SSCP analysis (Table 1). PCR was performed in 20 μl volumes containing 50–100 ng of genomic DNA template, 0.4 mM primer, 0.2 mM dNTPs, 2.5 units of *Taq* polymerase (Promega Inc., Madison, WI), 0.1 μl of α-^{32}P-dCTP (10 mci/ml, DuPont-NEN, Wilmington, DE) and 10% DMSO. Final magnesium chloride concentration was 1.5 mM for all amplifications. PCR conditions were as follows: initial denaturation at 95 °C for 5 min; 35 cycles of 94 °C for 30 s, 62–63 °C for 30 s, 72 °C for 30 s; plus a final extension at 72 °C for 10 min. For SSCP analysis, 1 μl of the final PCR product was added to 9 μl of loading buffer (95% formamide, 10 mM NaOH, 0.25% bromophenol blue, and 0.25% xylene cyanol), denatured at 94 °C for 5 min, and rapidly cooled on ice. One microliter of the denatured mixture was loaded on to 0.5 × MDE gel (BMA) and electrophoresis was performed (6 W at room temperature for 12 h). Following electrophoresis the gel was dried and exposed to X-ray film at − 80 °C for 12 h.

2.3. DNA sequencing

DNA samples exhibiting SSCP band shifts were reamplified using the same primers as the initial amplification. After purification (Qiagen Inc. Valencia, CA), reamplified PCR products were sequenced in both directions. Genomic DNAs from breast cancer cell lines were directly sequenced. Sequencing was performed by the OSU Biotechnology Center Sequencing Facility using the Dye-Terminator Ready Reaction Mix (Perkin–Elmer, Foster, CA) and an automated sequencer (ABI 371 PRISM).

3. Results and discussion

Loss of function mutations in tumor suppressor genes are common genetic alterations in breast cancer [16]. Previous reports from our laboratory have demonstrated that C/EBPD (C/EBPδ) plays a key role in the initiation and maintenance of mouse and human mammary epithelial cell G_0 growth arrest [7–11]. The human CEBPD gene maps to chromosome 8p11.1–p11.2 [13]. Alterations in 8p are common in human cancers [17]. Alterations in 8p occur with relatively high frequency in primary breast cancer and breast cancer cell lines [18–21]. Recently, Polyak and coworkers compared the global gene expression profile of normal human mammary epithelial cells to increasingly malignant breast cancer samples. The results demonstrated that C/EBPD expression decreased dramatically as human mammary epithelial cells progressed from normal to highly malignant breast cancer [12]. In addition to primary breast tumors, 8p losses have been demonstrated in 9/16 human breast cancer cell lines [20]. These results suggest that C/EBPD functions as a tumor suppressor gene in mammary epithelial cells.

In this study, mutational analysis of the C/EBPD gene was performed on 97 human breast cancer samples and 10 breast cell lines. The single exon, intronless, 807 base pair C/EBPD gene was amplified in four fragments. SSCP band shifts were detected from three samples (Fig. 1). DNA sequencing results are presented in Table 2. A silent C → T transition was detected at nucleotide 786 in two out of ninety-seven breast cancer samples (frequency: 2/97) (Fig. 1(b), Table 2). Aberrant SSCP mobility shifts were also detected from a third sample (Fig. 1(a)). DNA sequencing identified two nucleotide changes (Table 2), a silent C → T transition at nucleotide 753 and a G → T transversion at

Table 1
Primers for SSCP analysis of CEBPD

Fragment	Size (bp)	Annealing temp. (°C)	Name	Sequence
1	233	62	X39F 194R	5′-ACAGCCTCGCTTGGACGCAGAG-3′ 5′-AGTCGATGTAGGCGCTGAAGTC-3′
2	264	62	183F 447R	5′-AGCGCCTACATCGACTCCATGG-3′ 5′-CAAGCTCACCACGGTCTGTGC-3′
3	263	63	427F 690R	5′-GCACAGACCGTGGTGAGCTTG-3′ 5′-CGTTCTCAGCCGACAGCTCCA-3′
4	192	62	659F X850R	5′-AGCAGAAGTTGGTGGAGCTGTC-3′ 5′-GTATGGGTCCTTGCTGAGTCTC-3′

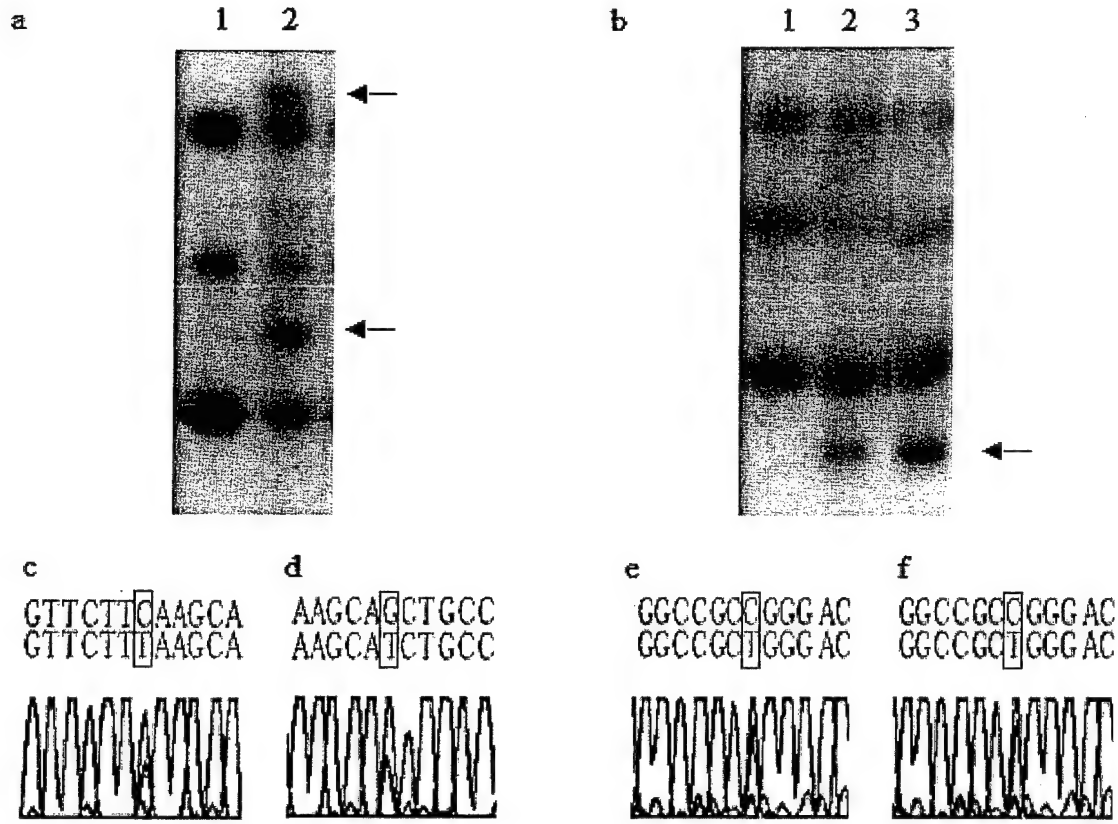

Fig. 1. PCR-SSCP and genomic sequence analysis of the CEBPD gene. (a) Lane 1, normal control; lane 2, breast cancer sample 1. (b) Lane 1, normal control; lane 2, breast cancer sample 2; lane 3, breast cancer sample 3. Arrows indicate SSCP mobility shifts. (c,d,e,f) Genomic sequence tracings detailing identifying specific base alterations in breast cancer samples.

nucleotide 759 resulting in the replacement of Glutamine for Histidine at codon 253. A nonsense mutation was found in the breast cancer cell line SUM-52PE using direct genomic DNA sequencing. An A → T transversion at nucleotide 538 resulted in a change from AAG to TAG at codon 180 and the insertion of a premature stop codon in the C/EBPD gene coding sequence.

The relatively low incidence of mutations in the C/EBPD gene observed in this study of breast cancer patients is consistent with two previous reports that have investigated C/EBPD gene structure in healthy normal controls and cancer patients. Angeloni et al recently reported a silent mutation (C → A transversion, nucleotide 306) in the human C/EBPD gene in lung cancer patients and Asian normal individuals [13]. In addition, Vegesan et al. reported a missense mutation in the C/EBPD gene at codon 177

Table 2
Sequence changes in CEBPD gene

Sample	Fragment	Nucleotide changed	Codon	Codon changed
1	4	TTC̄ to TTT̄	251	Silent mutation
	4	CAḠ to CAT̄	253	Gln to His
2	4	GCC̄ to GCT̄	262	Silent mutation
3	4	GCC̄ to GCT̄	262	Silent mutation
SUM-52PE	3	ĀAG to T̄AG	180	Lys to TAG

(A177G) in human lymphoma cell line Raji [6]. Although mutational inactivation of the C/EBPD gene is relatively uncommon in human cancers, C/EBPD gene expression decreases with increasing breast cancer malignancy [12]. This suggests that alternative mechanisms (promoter methylation, posttranscriptional control) may play a role in the decline in C/EBPD gene expression in human breast cancer. Preliminary studies in our lab indicate that the human breast cancer cell line SUM 52 PE does not express CEBPD (data not shown). Bisulfite treatment/genomic sequence analysis indicates that the SUM 52PE CEBPD gene promoter is heavily methylated (26/27 CpGs are methylated) (data not shown). Bisulfite treatment/genomic sequence analysis of 17 primary human breast cancer isolates exhibiting minimal CEBPD expression indicates that 6/17 (35%) exhibit selective CpG methylation in the CEBPD gene promoter (data not shown). The influence of methylation and chromatin structure on C/EBPD gene expression in primary human breast cancer is currently under investigation in our laboratory.

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P30 CA16058 (to OSU Comprehensive Cancer Center), National Cancer Institute, Bethesda, MD.

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Title: Progressive "Loss of Function" Alterations in the STAT3/C/EBPδ Growth Control Pathway: Comparative Analysis of Primary and Immortalized Human Mammary Epithelial Cells and Human Breast Cancer Cell Lines

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ABSTRACT

"Loss of function" alterations in growth inhibitory signal transduction pathways are common in cancer cells. In this study we show that growth arrest treatments - contact inhibition, growth factor withdrawal and growth inhibitory IL-6 family cytokines increase Stat3 phosphorylation (pSTAT3), increase CCAAT Enhancer Binding Protein δ (C/EBPδ) gene expression and induce growth arrest of primary, finite-lifespan human mammary epithelial cells and immortalized breast cell lines (MCF-10A and MCF-12A). In contrast, serum and growth factor withdrawal from human breast cancer cell lines (MCF-7, SK-BR-3, T-47D, MDA-MB-231 and SUM-44PE) did not increase pSTAT3 levels or C/EBPδ gene expression or induce growth arrest. In most breast cancer cell lines cytokine treatment increased pSTAT3 levels and C/EBPδ gene expression without inducing growth arrest. In the SUM-52PE breast cancer cell line cytokine treatment increased pSTAT3 levels but did not increase C/EBPδ gene expression or induce growth arrest. In the SUM-102PT breast cancer cell line pSTAT3 and C/EBPδ protein levels were constitutively elevated, however, SUM-102PT cells do not growth arrest in response growth arrest treatments. Finally, we show that activation of the pSTAT3/C/EBPδ growth control pathway is independent of estrogen receptor status. These results demonstrate that "loss of function" alterations in the pSTAT3/C/EBPδ growth inhibitory signal transduction pathway are common in human breast cancer cell lines and may play a role in the etiology or progression of breast cancer.

Keywords:
CCAAT/enhancer binding protein δ (C/EBPδ)
Cytokine
Growth arrest
Mammary epithelial cells
Signal Transducer and Activator of Transcription 3 (STAT3)

INTRODUCTION

Breast cancer is the second leading cause of cancer-related deaths in US women. Approximately 180,000 new cases of breast cancer are diagnosed each year in the US, and despite improved diagnostic and treatment modalities, annual breast cancer deaths exceed 40,000 [1]. Breast cancer development is a multi-step process, beginning with ductal or lobular atypical hyperplasia with progression to ductal or lobular carcinoma in situ (DCIS/LCIS), and eventually to invasive carcinoma and metastasis [2,3]. At the molecular level, breast cancer is characterized by the progressive accumulation of genetic errors in growth control genes and DNA repair genes, resulting in the characteristics associated with cancer cells - loss of contact inhibition, uncontrolled growth, immortalization and invasion [2]. One of the major goals in breast cancer research is to identify mammary epithelial cell growth-control pathways and to investigate "loss of function" changes in these pathways that may play a role in the development or progression of breast cancer.

We previously reported that C/EBPδ, a member of the CCAAT/Enhancer binding protein family of nuclear proteins, plays a key role in mouse mammary epithelial cell growth control in vitro and in vivo [4-10]. We found that serum and growth factor withdrawal or cytokine treatment of HC11 mouse mammary epithelial cells initiates a signal transduction cascade that includes activation of latent cytoplasmic Signal Transducer and Activator of Transcription 3 (STAT3), translocation of activated STAT3 dimers to the nucleus, STAT3 binding to consensus acute phase response elements (APREs) in the mouse C/EBPδ gene promoter, transactivation of the C/EBPδ gene, and growth arrest [9,10]. In addition, we showed that phosphorylated/activated STAT3 (pSTAT3) and C/EBPδ levels increase during mammary gland involution, and that virgin female C/EBPδ knockout mice exhibit aberrant mammary epithelial

cell proliferation and mammary gland ductal hyperplasia [11]. These results indicate that the STAT3/C/EBPδ growth-control signal transduction pathway plays a functionally significant role in normal mouse mammary epithelial cell growth regulation.

C/EBPδ is a member of the C/EBP family of nuclear proteins. Six C/EBP family members have been identified, including C/EBPα, C/EBPβ (also known as CRP2, NF-IL6, LAP, AGP/EBP, IL-6BP, and NF-M), C/EBPγ, C/EBPδ (CRP3, NF-IL6β, CELF), C/EBPε and C/EBPζ (CHOP10, GADD153) [12]. C/EBP family members are expressed in a tissue-specific manner and function in a wide range of cellular activities, including the regulation of cell growth and differentiation. C/EBPs function in the control of cell growth and differentiation by a variety of biochemical mechanisms including transcriptional activation of downstream target genes, such as GADD45γ, and protein-protein interactions with cell cycle regulatory proteins such as Rb, p21, cdk2 and cdk4 [13-16].

Recent reports indicate that alterations in the structure and expression of specific C/EBPs influence tumorigenesis. For example, C/EBPα plays a key role in granulocyte differentiation, and mutations in the C/EBPα gene are common in a subset of acute myeloid leukemia (AML) patients [17,18]. Alterations in C/EBPα structure and expression have also been reported in human lung cancer [19]. A second C/EBP family member, C/EBPβ, has been linked to carcinogen-induced skin tumorigenesis [20]. Polyak and coworkers recently utilized serial analysis of gene expression (SAGE) to investigate the molecular alterations associated with breast cancer progression. Their analysis of approximately 50,000 unique transcripts identified C/EBPδ as one of a small subset of genes (17 genes) that were consistently down-regulated with progression from normal human mammary epithelium to breast carcinoma [21].

The functional significance of C/EBPδ or the pSTAT3/C/EBPδ growth-control signal transduction pathway has not been systematically investigated in human mammary epithelial cells or human breast cancer cell lines. Therefore, the goals of the present study were to: (1) confirm the functional role of the pSTAT3/C/EBPδ growth-control signal transduction pathway in non-transformed human mammary epithelial cells (primary and immortalized), and (2) investigate potential "loss of function" alterations in the pSTAT3/C/EBPδ growth-control signal transduction pathway in human breast cancer cell lines. The results indicate that primary, finite-lifespan human mammary epithelial cells and immortalized, non-transformed human mammary epithelial cell lines exhibit growth inhibition in response to standard growth arrest conditions; i.e. contact inhibition, serum and growth factor withdrawal and cytokine treatment. This growth inhibition is characterized by the activation of the pSTAT3/C/EBPδ growth-control signal transduction pathway. Growth inhibition of MCF-12A cells was also demonstrated by direct over-expression of C/EBPδ using the clonogenic colony growth assay. In contrast, human breast cancer cell lines are not growth-inhibited by standard growth arrest conditions. Human breast cancer cell lines exhibit defects in STAT3 activation, C/EBPδ gene expression and/or C/EBPδ downstream gene function in response to growth arrest conditions. Finally, we show that the pSTAT3/C/EBPδ growth control signal transduction pathway is independent of estrogen receptor status. These results demonstrate that "loss of function" alterations in the STAT3/C/EBPδ growth-arrest signal transduction pathway are common in human breast cancer cell lines. These results indicate that loss of function alterations in the pSTAT3/C/EBPδ growth-control signal transduction pathway are common in human breast cancer cell lines and may play a role in the development or progression of clinical breast cancer.

MATERIALS/METHODS

Cell Culture. Cell culture media and components were purchased from Invitrogen/Life Technologies, Inc. and Sigma. Cells were purchased from American Type Culture Collection (ATCC, Manassas, VA) and maintained in ATCC recommended phenol red free media with the addition of 100U/ml penicillin:100 μg/ml streptomycin and 500 ng/ml Fungizone (Invitrogen) unless otherwise specified. MCF-10A and MCF-12A cell lines were growth arrested in 1:1 DMEM/F-12 with .1% horse serum. T-47D cell line was growth arrested in RPMI 1640 (Sigma) with .1% FBS. MCF-7 cell line was cultured in DMEM without phenol red (Sigma) with 10 μg/ml bovine insulin, 4mM L-Glutamine, 10 ng/ml human EGF, 100 U/ml penicillin:100 μg/ml streptomycin, 500 ng/ml Fungizone, and 10% FBS. Growth arrest media contained only .1% FBS. MDA-MB-231 cell line was purchased from ATCC and cultured in Minimum Essential Eagle's Media (with Earle's salts and non-essential amino acids, without L-glutamine, phenol red and sodium bicarbonate) (Sigma), 2 mM L-glutamine, 1.5 g/liter sodium bicarbonate, 1 mM sodium pyruvate, 100 U/ml penicillin:100 μg/ml streptomycin, 500 ng/ml Fungizone, and 10% heat-inactivated FBS. Growth arrest media contained only .1% heat-inactivated FBS. SK-BR-3 cell line was growth arrested in .1% FBS. SUM44PE, SUM52PE, and SUM102PT cell lines were graciously donated by Dr. Stephen Ethier of the University of Michigan Breast Cancer Cell/Tissue Bank and Data Base and were cultured in Serum Free Ham's F-12 media with 5 μg/ml bovine insulin, 1 μg/ml hydrocortisone, 1 g/liter bovine serum albumin (JRH Biosciences), 5 mM ethanolamine (Sigma), 10mM HEPES, 5 μg/ml transferrin (Sigma), 10 μM 3,3',5-triiodo-L-thyronine (Sigma), 50 μM sodium selenite (Sigma), 5 μg/ml gentamycin, and 500 ng/ml Fungizone. Growth arrest media lacked the insulin and hydrocortisone. HUMAN MAMMARY EPITHELIAL CELL LINES (HMECs) - HMECs were purchased from Clonetics

and cultured in Mammary Epithelial Basal Medium (MEBM) with the addition of 13 mg/ml BPE, 10 μg/ml hEGF, 5 mg/ml Insulin, 0.5 mg/ml Hydrocortisone, 50 mg/ml Gentamicin, and 50 μg/ml Amphotericin-B, all supplied by Clonetics. Growth arrest media contained only the Gentamicin and Amphotericin-B.

Growth Arrest and Cytokine Addition Studies. Near confluent (70%) cells were rinsed and then cultured in serum and growth factor deprived media (specified above for each cell line) for designated time periods. Human IL-6 and Oncostatin M (OSM) were obtained from Peprotech, reconstituted in sterile water and were added at a dose of 50 ng/ml. Cytokine addition studies were performed similar to the growth arrest studies by incubating near confluent cells in either growth arrest or growth media for the designated time periods with and without the addition of IL-6/OSM.

[^{3}H]Thymidine/Cell Cycle Experiments. Cell cycle experiments were conducted on cells cultured in 12-well plates. Cells were plated at ~50,000 cells/well and allowed to reach 70% confluence before initiating growth arrest. [^{3}H]Thymidine was added two hours prior to collection. Media containing [^{3}H]thymidine was removed and cells were washed in the wells with 10% trichloroacetic acid three times at 4°C (1 x 10 minutes, 2 x 5 minutes). Cells were dissolved in 250 μl 1M NaOH/1% SDS and the solution transferred to a vial containing 5 ml scintillation fluid. Samples were dissolved and then counted using a liquid scintillation counter.

Northern Blot Analysis. Total RNA was isolated using RNAzol B (Tel-Test, Inc.), run on 1.2% agarose gels and transferred to Duralon UV membrane followed by UV crosslinking. Filters were hybridized for a minimum of 2 hours. DNA probes were labeled with [alpha-^{32}P]dCTP using a Random Primers Labeling Kit (Invitrogen), added to the hybridization mixture and allowed to hybridize a minimum of two hours. C/EBPδ probe was a 700 bp fragment isolated

from Research Genetics Clone 82850. TTK (Threonine Tyrosine Kinase - ATCC 80028) was used as an S-phase/growth marker. Human Growth Arrest Specific Protein 1 (GAS1) probe was isolated from ATCC Clone 959480. Cyclophylin (CP) was used as a loading control.

Western Blot Analysis. Whole cell protein was isolated using a whole cell lysis buffer containing 20 mM Tris pH 8.0, 137 mM NaCl, 10% glycerol, 1% IGEPAL, .1% SDS, .5% sodium deoxycholate, 2 mM EDTA, 1mM PMSF, 1X complete protease (Boehringer Mannheim), 1mM NaF, 1 mM $NaVO_3$, 1mM Na_2MoO_4, 100 nM okadaic acid. Proteins were run on precast BioRad 10-12% Tris-HCl gels and were transferred to PVDF membrane. Rabbit polyclonal C/EBPδ, C/EBPβ, and β-Actin antibodies were obtained from Santa Cruz. Rabbit polyclonal STAT3 and Phospho-STAT3 were obtained from Cell Signaling. Westerns were blocked in 10% milk with primary and secondary antibody addition in 5% milk. Signal was developed using ECL and ECL Plus detection systems (Amersham Biosciences).

Clonogenic Assay. Transfections were performed using Lipofectamine Plus (Invitrogen). MCF-12A cells were transfected with 1 μg of PCDNA3pcDNA3 or PCDNA3pcDNA3-full length C/EBPδ constructs. Transfected cells were selected for with media containing 400 μg/ml G418 (Geneticin). Colonies were stained with .5% crystal violet in 20% methanol.

Estrogen and Tamoxifen Assays. MCF-7 cells plated at 70% confluence were growth arrested for 72 hours in DMEM without phenol red with the addition of .1% charcoal-stripped fetal calf serum (Life Technologies). 17β-Estradiol (Sigma) was added at 100 pM to growth arrested cells with or without the addition of 100 nM 4-Hydroxytamoxifen (Sigma).

Statistical Analysis. Statistical analysis was performed using one-way Analysis of Variance (ANOVA) and Bonferroni posttest analysis.

RESULTS

Contact Inhibition, Serum and Growth Factor Withdrawal and Oncostatin M Addition Activate STAT3, Induce C/EBPδ mRNA and Protein and Result in Growth Arrest of Finite-lifespan Human Mammary Epithelial Cells (HMECs)

To determine whether C/EBPδ gene expression was induced during G_0 growth arrest of human mammary epithelial cells, finite-lifespan human mammary epithelial cells (HMECs) were growth arrested by three standard methods: cellular confluence, serum and growth factor withdrawal, and growth-inhibitory cytokine (OSM) addition. The initial experiment assessed the influence of cellular confluence, serum and growth factor withdrawal, and OSM treatment on HMEC C/EBPδ mRNA levels. Compared to growing (untreated) cultures, each growth arrest treatment significantly induced (2- 4 fold) C/EBPδ mRNA levels (Fig. 1a, lane 1 vs. lanes 2-10).

We next investigated the influence of contact inhibition, serum and growth factor withdrawal and OSM treatment on STAT3 phosphorylation, C/EBPδ gene expression and primary HMEC growth arrest. STAT3 protein levels were constitutive in all HMEC samples regardless of treatment (Fig. 1b). Compared to growing (untreated) cultures, each growth arrest treatment resulted in increased levels of phosphorylated STAT3 (pSTAT3) (Fig. 1b, lane 1 vs. lanes 2-10). The highest levels of pSTAT3 were detected in OSM-treated HMECs (Fig. 1b, lanes 7-10). C/EBPδ protein was only detected in growth-arrested cultures that exhibited STAT3 phosphorylation (Fig. 1b, lanes 2-10). The relative induction of C/EBPδ protein in response to the individual growth-arrest treatments paralleled the relative induction of C/EBPδ mRNA levels (Fig. 1a, lanes 2-10). The levels of p27, a growth-arrest specific protein, also increased following the three growth-arrest treatments (Figure 1b, lane 1 (growing) vs. lanes 2-10).

The influence of growing and growth-arrest treatments was assessed by [^{3}H]thymidine incorporation (Fig. 1c). [^{3}H]Thymidine incorporation increased in growing (GR) HMECs cultured for 24 and 48 hours in complete growth media (Fig. 1c). Addition of OSM to HMECs in complete growth media (GR-OSM) significantly reduced [^{3}H]thymidine incorporation at both 24 and 48 hour time points ($p<0.0001$) compared to untreated HMECs in complete growth media (GR). Serum and growth factor withdrawal alone (growth arrest (GA)) and in combination with OSM treatment (GA-OSM) significantly reduced HMEC [^{3}H]thymidine incorporation compared to growing cultures ($p<0.0001$). These results indicate that contact inhibition, serum and growth factor withdrawal and OSM treatment induce cell cycle exit and growth inhibition of primary, finite-lifespan HMECs. Growth-inhibited HMECs exhibit increased pSTAT3 levels and increased C/EBPδ gene expression, consistent with previous reports in HC11 mouse mammary epithelial cells [9,10].

Serum and Growth Factor Withdrawal and IL-6 Family Cytokine Addition Increases pSTAT3 levels, Increases C/EBPδ mRNA and Protein levels, and Initiates Growth Arrest in Immortalized Non-tumorigenic Human Mammary Epithelial Cell Lines MCF-10A and MCF-12A

Previous reports by Stampfer and coworkers have correlated specific "loss of function" alterations in growth control genes with human mammary epithelial cell immortalization [22-25]. To further investigate the association between STAT3 activation, C/EBPδ induction and the initiation of G_0 growth arrest, we utilized two immortalized, but non-tumorigenic human mammary epithelial cell lines, MCF-10A and MCF-12A. MCF-10A and MCF-12A cell lines exhibit near diploid karyotypes and have undergone relatively few chromosome rearrangements [26,27].

Serum and growth factor withdrawal alone, and serum and growth factor withdrawal plus cytokine treatment (IL-6 or OSM) from near confluent MCF-10A and MCF-12A cells induced changes in gene expression consistent with cell cycle exit and G_0 growth arrest. TTK mRNA levels (S phase marker) declined from "Growing" (Fig. 2a,b, lane 1) to 48 hour G_0 growth arrest/ cytokine treatment (Fig. 2a,b, lane 1 vs. 2-10). In addition, gas1 mRNA levels (G_0 growth arrest marker) increased from "Growing" (Fig. 2a,b, lane 1) to 48 hour G_0 growth arrest/cytokine treatment (Fig. 2a,b, lane 1 vs. 2-10). We previously reported that growth arrest-induced changes in HC11 mouse mammary epithelial cell C/EBPδ mRNA levels parallel changes in gas1 mRNA levels [4,7]. In this report, we show that C/EBPδ mRNA levels in G_0 growth arrest/cytokine-treated MCF-10A and MCF-12A human mammary epithelial cells are induced 2-5 fold over C/EBPδ mRNA levels in growing cells (Fig. 2a,b, lane1 vs. lanes 2-10).

C/EBPδ protein levels increased 2-3 fold by 12 hours of serum and growth factor withdrawal and remained elevated through the 48 hour treatment period in both the MCF-10A and MCF-12A (Fig. 2c,d, lanes 1-3). Addition of OSM or IL-6 in conjunction with serum and growth factor withdrawal increased C/EBPδ protein levels 4-fold over serum and growth factor withdrawal alone by 12 hours (Fig. 2c,d, lanes 4,7). C/EBPδ protein levels remained elevated at both 24 and 48 hour timepoints (Fig 2c,d, lanes 5,6,8,9). STAT3 was constitutively expressed in both cell lines, however, phosphorylated STAT3 (pSTAT3) was only present in growth-arrested (Fig.2c,d, lanes 1-3) and cytokine-treated (Fig. 2c,d, lanes 4-9) cells. p27 protein levels increased following both serum and growth factor withdrawal and cytokine treatment, consistent with a growth-arrest state. C/EBPβ protein was constitutively expressed regardless of growth condition. These results suggest a novel, growth arrest-specific role for C/EBPδ and relatively constitutive role for C/EBPβ in mammary epithelial cell biology.

[^{3}H]Thymidine incorporation decreased following serum and growth factor withdrawal and cytokine treatment compared to untreated growing controls (Fig. 2e,f). Analysis of serum and growth factor withdrawal/cytokine-treated cultures by flow cytometry confirmed the increase of cells in G_0/G_1 (37% in growing cells vs. 75% in serum deprived/cytokine treated cells) (data not shown). These results demonstrate that increased pSTAT3 levels and increased C/EBPδ gene expression are linked to the G_0 growth inhibition in immortalized human mammary epithelial cell lines consistent with the results from studies with finite lifespan HMECs (Fig. 1, above).

Serum and Growth Factor Withdrawal Plus IL-6 family Cytokines Induces Variable Increases in pSTAT3 and C/EBPδ mRNA and Protein Levels, but does not Growth Arrest Human Breast Cancer Cell Lines MCF-7, MDA-MB-231, SK-BR-3 and T-47D

We next investigated the activation of the pSTAT3/C/EBPδ growth control signal transduction pathway in human breast cancer cell lines under growth arrest conditions. Four human breast cancer cell lines were investigated in the initial experiments – MCF-7, MDA-MB-231, SK-BR-3, and T-47D. Human breast cancer cell lines express detectable levels of TTK mRNA when cultured in complete growth media ("Growing") (Fig. 3a, lanes 1,5,9,13). Withdrawal of serum and growth factors (growth arrest (GA)) or GA plus cytokine treatment for 24 hours did not result in a consistent decline in TTK mRNA levels. This suggests that standard growth arrest treatments do not adversely affect breast cancer cell line proliferation in the short term. The only cell line that exhibited a significant decline in TTK mRNA levels was the GA plus OSM treated MDA-MB-231 cell line (Fig. 3a, lane 3). In contrast to results from the non-transformed human mammary epithelial cell lines (Figs. 1 and 2), removal of serum and growth

factors (growth arrest (GA)) did not significantly induce C/EBPδ mRNA in the four breast cancer cell lines (Fig. 3a, lanes 2,6,10,14). Although C/EBPδ mRNA levels were not induced in the human breast cancer cell lines by GA conditions alone, C/EBPδ mRNA levels did increase in most GA plus OSM or GA plus IL-6 treated human breast cancer cell lines (Fig. 3a, lanes 3,4,7,8,11,12,15,16).

Consistent with the mRNA results, removal of serum and growth factors (growth arrest treatment) did not significantly induce C/EBPδ protein levels in the four breast cancer cell lines (Fig. 3b, lanes 2-4). However, GA plus OSM or GA plus IL-6 treatment did result in an increase in C/EBPδ protein levels (Fig. 3b, lanes 5-7 and 8-10). STAT3 was constitutively expressed in all four cell lines regardless of treatment. Phosphorylated (activated) STAT3 (pSTAT3) was not detected in growing or growth arrested human breast cancer cell lines (Fig. 3b, lanes 1-4). pSTAT3 was detected in GA plus OSM treated MDA-MB-231 cells, but not in GA plus IL-6 treated MDA-MB-231 cells (Fig. 3b, lanes 5-7 vs. 8-10). pSTAT3 levels were also higher in GA plus OSM treated T-47D cells compared to GA plus IL-6 treated T-47D cells (Fig. 3b, lanes 5-7 vs. 8-10). In SK-BR-3 cells, pSTAT3 levels were higher following GA plus IL-6 treatment compared to GA plus OSM treatment (Fig. 3b, lanes 8-10 vs. 5-7). MCF-7 cells exhibited modest pSTAT3 induction following GA plus OSM treatment and little or no pSTAT3 induction following GA plus IL-6 treatment (Fig. 3b, lanes 8-10).

[^{3}H]Thymidine incorporation into GA and GA plus cytokine treated human breast cancer cell lines was reduced in SK-BR-3 and MCF-7 cells by 48 hours, but was similar to untreated growing cells in MDA-MB-231 and T-47D cells (data not shown). This suggests the growth arrest pathways initiated by GA and GA plus cytokine treatments are disregulated in human breast cancer cell lines, or that human breast cancer cell lines produce autocrine growth factors

enabling them to override growth arrest signals. Evidence exists to support both scenarios. Disruptions in growth inhibitory pathways have been reported in human breast cancer cell lines. For example, the Cdk inhibitor, p27, is expressed at elevated levels in both the MCF-7 and T-47D cell lines without inhibiting cell cycle progression [28]. Constitutively activated Stat3 has also been associated with oncogenesis [29,30]. In addition, several reports indicate that human breast cancer cell lines produce autocrine growth factors capable of maintaining cell growth in the absence of exogenous growth factors [31].

Growth Arrest and C/EBPδ mRNA and Protein Induction are Variable With Growth Arrest and IL-6 Family Cytokine Treatment in the SUM Human Breast Cancer Cell Lines

Comparative analysis of the results from the finite-lifespan HMECs, immortalized human breast derived cell lines and human breast cancer cell lines suggests a progressive disruption of the pSTAT3/C/EBPδ growth arrest pathway with progressive alterations in cell growth phenotype. We next investigated the pSTAT3/C/EBPδ growth arrest pathway in the three human breast cancer cell lines recently developed at the University of Michigan; SUM 44-PE, SUM 52-PE and SUM 102-PT [32-35]. The influence of serum and growth factor withdrawal-induced growth arrest (GA) on C/EBPδ mRNA levels was variable among the three SUM breast cancer cell lines (Fig. 4a). Serum and growth factor withdrawal resulted in low level induction of C/EBPδ mRNA in the SUM 44-PE (Fig. 4a, lanes 1-5) and no induction of C/EBPδ mRNA in the SUM 52-PE cell line (Fig. 4a, lanes 6-9). In contrast, C/EBPδ mRNA levels were elevated in the SUM 102-PT regardless of culture conditions (Fig. 4a, lanes 10-14). C/EBPδ protein levels correlated with mRNA levels for each cell line. That is, low levels of C/EBPδ protein were detected in serum and growth factor withdrawn (Growth Arrest (GA) treated) SUM 44-PE cells

(Fig. 4b, lanes 1-2) and no C/EBPδ protein was detected in GA-treated SUM 52-PE cells (Fig. 4b, lanes 3-4). Constitutively elevated levels of C/EBPδ protein were detected in SUM 102-PT cells (Fig. 4b, lanes 5-6). The addition of OSM or IL-6 to complete growth media increased STAT3 phosphorylation, C/EBPδ mRNA and C/EBPδ protein levels in SUM 44-PE cells (Fig. 4c, lanes 1-3) and also in SUM 102-PT cells (Fig. 4c, lanes 7-9). OSM and IL-6 treatment increased STAT3 phosphorylation in the SUM 52-PE cells, however, no C/EBPδ mRNA or protein was detected (Fig. 4c, lanes 4-6). Even though GA treatments increased pSTAT3 levels in all three SUM cell lines and increased C/EBPδ gene expression in the SUM 44-PE and SUM 102-PT cell lines, none of the SUM cell lines growth arrested in response to serum and growth factor withdrawal, and only the SUM 44-PE responded to OSM and IL-6 treatment (Figure 4d).

C/EBPδ Over-expression Significantly Decreases MCF-12A Colony Formation

To further evaluate the role of C/EBPδ as a growth suppressor, we expressed C/EBPδ in MCF-12A cells and evaluated colony formation in a clonogenic assay (Figure 5). Ectopic C/EBPδ expression decreased colony formation by 80% compared to vector control (pcDNA3) transfected cells. These results support the growth inhibitory activity of C/EBPδ in human mammary epithelial cells.

Stat3/C/EBPδ Growth Inhibition is Independent of Estrogen Receptor Status

To determine whether the growth suppressive role of C/EBPδ was related to estrogen receptor status, the growth arrest response of four breast cancer cell lines, MCF-7, T-47D, SK-BR-3 and MDA-MB-231, to serum and growth factor withdrawal and cytokine addition was correlated with estrogen receptor status (Figure 6a). One ER+ and one ER- cell line responded

to Stat3/C/EBPδ growth inhibition (MCF-7 and SK-BR-3). One ER+ and one ER- cell line failed to respond (T-47D and MDA-MB-231). This suggests that functionality of the Stat3/C/EBPδ growth inhibitory pathway does not depend on the presence or absence of the estrogen receptor. To confirm these findings, MCF-7 cells, which are known to be ER+, were growth arrested in charcoal-stripped serum for 72 hours, followed by the addition of 17β-estradiol to induce a pure 'estrogen-induced' growth (Figure 6b). Estrogen-induced growth was then suppressed by the addition of 4-hydroxytamoxifen (4-OHT) (Figure 6c). C/EBPδ mRNA levels were not decreased by the addition of 17β-estradiol and did not increase after 4-OHT treatment (Figure 6d), suggesting that estrogen receptor antagonists do not activate the Stat3/C/EBPδ growth inhibitory pathway. Studies with the pure anti-estrogen ICI 182,780 produced similar results (data not shown). These studies suggest that the C/EBPδ growth inhibitory pathway is not estrogen-regulated. Hence, determining methods for activation of the Stat3/C/EBPδ pathway could provide alternative treatment options for patients with unresponsive ER+ breast cancer and those with ER- breast cancer.

DISCUSSION

The goal of the present study was to investigate the role of C/EBPδ and its principal transcriptional regulator, pSTAT3, in the growth arrest response of human mammary epithelial cells. In the initial studies reported here, we investigated the activation of the pSTAT3/C/EBPδ signal transduction pathway in primary, finite-lifespan HMECs. Stampfer and coworkers have extensively investigated growth regulatory pathways in primary HMECs [22-25]. Their studies have demonstrated that growth factor receptor blockade and TGFβ treatment induce G_0 growth arrest in primary, finite-lifespan HMECs [23]. In the present studies, we showed that contact

inhibition, serum and growth withdrawal and IL-6 family cytokine treatment also induce G_0 growth arrest of primary, finite-lifespan HMECs (Fig. 1a-c). Our data also demonstrate that primary HMEC growth arrest is associated with activation of the pSTAT3/C/EBPδ signal transduction pathway. These results are consistent with previous results from our lab demonstrating a role for the pSTAT3/C/EBPδ signal transduction pathway in G_0 growth arrest of HC11 mouse mammary epithelial cells [7,9]. These results are also consistent with previous in vivo studies in which we demonstrated that nulliparous C/EBPδ knockout female mice exhibit aberrant mammary epithelial cell proliferation [6,11]. These collective results all support a growth regulatory role for the pSTAT3/C/EBPδ signal transduction pathway in normal mammary epithelial cell biology.

Although our studies did not directly investigate the conversion of primary HMECs to immortalized HMECs, we did investigate the growth arrest response of two immortalized HMEC cell lines, MCF-10A and MCF-12A. Both MCF-10A and MCF-12A cell lines growth arrested in response to growth arrest (GA) treatments - serum and growth factor withdrawal alone or serum and growth factor withdrawal plus IL-6 family cytokines (Fig. 2e,f). In addition, growth-arrested MCF-10A and MCF-12A cells exhibited increased pSTAT3 levels, increased C/EBPδ mRNA and increased C/EBPδ protein levels (Fig. 2a-d). It is of interest that the immortalized MCF-10A and MCF-12A cell lines were not growth arrested by IL-6 family cytokine treatment when cultured in complete growth media, despite exhibiting increased pSTAT3 levels and increased C/EBPδ gene expression (data not shown). Stampfer and coworkers have demonstrated that one of the hallmarks of primary HMEC immortalization is loss of growth inhibition in response to specific growth arrest treatments, such as TGFβ [23]. These results indicate that growth in the

presence IL-6 family cytokines, like growth in the presence of TGFβ, may be one of the growth control functions lost with HMEC immortalization.

Serum and growth factor withdrawal did not significantly activate the pSTAT3/C/EBPδ signal transduction pathway and only growth arrested 2/9 human breast cancer cell lines studied (Figures 3, 4 & data not shown). Although IL-6 family cytokines consistently induced the pSTAT3/C/EBPδ signal transduction pathway, IL-6 family cytokine treatment only induced growth arrest in 3/9 cell lines studied (Figures 3, 4 & data not shown). These results suggest that the pSTAT3/C/EBPδ signal transduction pathway and its role in mammary epithelial cell growth control become increasingly dysfunctional with the progression from immortalized to transformed cell lines. Autocrine growth factors produced by human breast cancer cell lines may also contribute to the loss of growth control by overriding cytokine-induced growth inhibitory signals. These results are consistent with a previous report demonstrating that IL-6 treatment of MCF-7 cells induced C/EBPδ, but did not induce growth arrest [36]. Although our short term (48-72 hours) experiments did not result in cytokine-induced growth inhibition, long term (10 day) IL-6 treatment has been reported to result in growth inhibition of T-47D cells [37]. It is possible that long-term cytokine treatments may result in partial growth inhibition or may induce programmed cell death.

The growth arrest response of the SUM-44PE cell line, a recently developed human breast cancer cell line, was similar to the MCF-7 and T-47D human breast cancer cell lines. SUM-44PE cells exhibited activation of the pSTAT3/C/EBPδ signal transduction pathway in response to growth arrest treatments, did not growth arrest with serum and growth factor withdrawal, but did growth arrest with cytokine addition (Fig. 4a-d). These results suggest that the pSTAT3/C/EBPδ signal transduction pathway can be activated in human breast cancer cell

lines, but loss of function of downstream effector gene products may prevent full activation of the growth arrest program. Results from the SUM-52PE provided the first direct evidence of a specific defect in C/EBPδ transcription/translation in a human breast cancer cell line. Previous reports have shown that SUM-52PE human breast cancer cells contain an amplified FGFR2 gene and over express the FGFR2 mRNA at levels 40 fold higher than normal breast-derived cells. FGFR2 over-expression, which may play a role in enhanced breast cancer growth, has been detected in 5-10% of breast cancer patients [35].

Our results identified another genetic alteration in SUM-52PE cells that may result in enhanced or aberrant growth. Growth arrest treatments increase pSTAT3 levels, but do not induce C/EBPδ gene expression or result in growth arrest of SUM-52PE cells (Fig. 4a-d). These results suggest a functional defect in pSTAT3 activation of the C/EBPδ gene promoter. Loss of growth suppressor gene expression due to promoter methylation is common in cancer cells [38]. Methylation analysis studies demonstrated that 26/27 CpG dinucleotides in the SUM-52PE C/EBPδ gene promoter were methylated. We have subsequently found that SUM-52PE cells are capable of activating a C/EBPδ gene promoter-luciferase reporter construct (Tang et al., manuscript in preparation). This indicates that the transcriptional machinery required to activate the C/EBPδ gene promoter is intact in SUM-52PE cells, but that promoter hypermethylation of the endogenous C/EBPδ gene promoter is responsible for loss of C/EBPδ gene expression (data not shown). Preliminary treatment of SUM-52PE cells with 5-azacytidine has reversed the extensive C/EBPδ gene promoter CpG methylation resulting in minimal C/EBPδ mRNA expression (data not shown). Sequence analysis has also revealed a nonsense mutation in the SUM-52-PE C/EBPδ coding region [39]. Nonsense-mediated decay of the SUM-52PE C/EBPδ mRNA may result in a highly unstable mRNA that is undetectable by Northern blot but

is detected by RT-PCR. Finally, ectopic expression of C/EBPδ reduces SUM-52PE colony formation similar to the MCF-12A, indicating that restoration of C/EBPδ gene expression induces growth inhibition of the SUM-52PE cell line (Figure 5 and data not shown).

In contrast to the SUM-52PE cell line in which the C/EBPδ gene promoter is silenced, C/EBPδ mRNA and protein levels are constitutively elevated in the SUM 102-PT cell line regardless of growth conditions (Fig. 4a-c). This observation is consistent with previous reports demonstrating constitutively elevated pSTAT3 levels in SUM 102-PT cells [34] and data from our lab and others demonstrating that pSTAT3 activates C/EBPδ gene transcription [9,40]. The constitutive activation of the pSTAT3/C/EBPδ signal transduction pathway in exponentially growing SUM102-PT cells suggests that the regulation and growth-inhibitory function of this pathway are defective. The mechanism of this loss of function is unknown, but is currently under investigation.

Hormonal control and the abundance of estrogen/progesterone receptors are also important factors in cell cycle progression of breast cancer cells. Receptor positive cells growth arrest when treated with receptor antagonists while receptor negative cells do not. Estrogen (estradiol), a potent mitogen, has been shown to upregulate p53 and induce hyperphosphorylation of Rb [41]. Synthetic anti-estrogens, such as tamoxifen and 4-hydroxytamoxifen, induce G_0/G_1 growth arrest in ER+ breast cancer cells by competitively binding the estrogen receptor but also have been shown to inhibit proliferation in certain ER- cells by the induction of p21 and p27 and accumulation of hypo-phosphorylated Rb [42,43]. In this study, induction of the pStat3/C/EBPδ inhibitory pathway and G_0 growth arrest were not affected by estrogen receptor status (Figure 6a) – both ER+ and ER- cells growth arrested or failed to growth arrest with serum and growth factor withdrawal or cytokine addition. Estrogen-induced growth in MCF-7 cells was inhibited

by tamoxifen addition (Figure 6b, c), as has been previously described [44], however tamoxifen-induced growth arrest did not increase C/EBPδ mRNA levels (Figure 6d). The pStat3/C/EBPδ growth inhibitory pathway appears to initiate growth arrest independently of the ER pathway. Hence, treatments which induce growth arrest by activation of the pStat3/C/EBPδ pathway could be potential therapies in unresponsive ER+ or ER- breast cancer patients.

In conclusion, this study demonstrates that the pSTAT3/C/EBPδ signal transduction pathway plays an important role in the regulated growth arrest of human mammary epithelial cells. This data is consistent with previous work from our laboratory in which activation of the pSTAT3/C/EBPδ signal transduction pathway has been shown to regulate mouse mammary epithelial cell growth arrest in vivo and in vitro despite differences in the location and orientation of critical regulatory elements (Sp1 and STAT binding sites) between the mouse and human C/EBPδ promoter [9]. These results demonstrate that the function of the pSTAT3/C/EBPδ signal transduction pathway is progressively disrupted in immortalized and transformed mammary epithelial cells. This "loss of function" pattern in the pSTAT3/C/EBPδ signal transduction pathway is consistent with recent SAGE studies by Polyak and coworkers in which loss of C/EBPδ gene expression was correlated with increased breast cancer progression [45]. Studies are ongoing in our laboratory to further investigate the expression of C/EBPδ in breast cancer patients. In addition, C/EBPδ gene promoter analysis and characterization of the downstream effectors of the pSTAT3/C/EBPδ signal transduction pathway are in progress.

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LEGENDS TO FIGURES

Figure 1: Induction of C/EBPδ mRNA and protein in primary HMECs following three methods of growth arrest – cellular confluence, serum and growth factor withdrawal and OSM treatment. **a)** RNA was isolated from HMECs under the following conditions and probed for C/EBPδ and CP (cyclophylin): 1) Growing (50% confluent), 2-4) Cells maintained in growth media attaining cellular confluence for 24, 48, and 72 hours, 5-6) Near confluent plates 24 and 48 hours following serum and growth factor withdrawal, 7-8) Near confluent plates in growth media with 50 ng/ml OSM for 24 and 48 hours, and 9-10) Near confluent plates in growth arrest media containing 50 ng/ml OSM for 24 and 48 hours. **b)** Whole cell protein was isolated from HMECs under the conditions described above in a). Western blots were performed and probed with primary antibodies to STAT3, phosphoSTAT3, C/EBPδ, p27 and β-Actin (loading control). **c)** [^{3}H]Thymidine incorporation of HMECs initially plated at 35% confluence under the following conditions: 1) Growth media (GR), 2) Growth media containing 50 ng/ml OSM (GR-OSM), 3) Growth arrest media (GA), and 4) Growth arrest media containing 50 ng/ml OSM (GA-OSM).

Figure 2: Induction of C/EBPδ mRNA and protein and decrease in [^{3}H]thymidine incorporation following serum and growth factor withdrawal and IL-6 family cytokine addition of MCF-10A and MCF-12A human mammary epithelial cell lines. Near confluent plates were switched from complete growth media to media containing only .1%FBS and no additional growth factors with and without 50 ng/ml IL-6 or OSM. **a & b)** RNA was isolated from growing cells and at 12, 24, and 48 hours of growth arrest with or without IL-6 and OSM. Northern blots were analyzed for

C/EBPδ expression and cell cycle markers TTK (threonine tyrosine kinase) and GAS1 (growth arrest specific 1). CP (cyclophylin) was used as a loading control. **c & d)** Near confluent plates were growth-arrested with and without IL-6 and OSM, whole cell protein isolated at 12, 24, and 48 hour timepoints, and western blotted. Westerns were probed with primary antibodies to STAT3, phosphoSTAT3, C/EBPδ, C/EBPβ, CDK inhibitor p27 and β-Actin. **e & f)** [^{3}H]Thymidine incorporation of MCF-10A and MCF-12A cells in growth media (GR), growth arrest media (GA), growth arrest media with 50 ng/ml OSM (GA-OSM) and growth arrest media with 50 ng/ml IL-6 (GA-IL6).

Figure 3: **a)** C/EBPδ mRNA induction following growth arrest and IL-6/OSM treatment of human breast cancer cell lines, MDA-MB-231, T-47D, SK-BR-3, and MCF-7. Near confluent plates were deprived of serum and growth factors (GA) with and without the addition of 50 ng/ml IL-6 (GA-IL6) or OSM (GA-OSM) for 24 hours. RNA was isolated, Northern blotted, and analyzed for C/EBPδ and growth marker TTK expression. **b)** STAT3 phosphorylation and C/EBPδ protein induction in breast cancer cell lines, MDA-MB-231, T-47D, SK-BR-3, and MCF-7, following serum and growth factor withdrawal and IL-6/OSM treatment. Near confluent plates were deprived of serum and growth factors with and without the addition of 50 ng/ml IL-6 (GA-IL6) or OSM (GA-OSM) for 12, 24, and 48 hours and whole cell protein isolated. Western blots were probed with primary antibodies to STAT3, phosphoSTAT3, C/EBPδ, p27 and β-Actin.

Figure 4: Variable induction of C/EBPδ following growth factor withdrawal in SUM (University of Michigan developed) breast cancer cell lines. **a)** Near confluent plates of three SUM cell lines, SUM-44PE, SUM-52PE, and SUM-102PT, were growth arrested by growth factor withdrawal. RNA was isolated at 24, 48 and 72 hours and Northern blotted. Northern blots

were analyzed for C/EBPδ and cell cycle growth marker TTK. CP was used as a loading control. **b)** Near confluent plates were growth arrested for 96 hours by growth factor withdrawal and RNA and whole cell protein isolated and Northern/Western blotted. **c)** Induction of C/EBPδ following IL-6 and OSM treatment of SUM breast cancer cell lines. Near confluent plates were allowed to remain in growth media or were switched to growth media containing either 50 ng/ml IL-6 (GR-IL6) or 50 ng/ml OSM (GR-OSM). RNA and protein were isolated and run on Northern and Western blots. Northerns were probed with C/EBPδ and growth marker TTK. Westerns were probed with primary antibodies to STAT3, phosphoSTAT3, C/EBPδ and β-Actin. **d)** Forty-eight hour [^{3}H]thymidine incorporation of cells cultured in growth media (GR), growth arrest media (GA), growth arrest media with 50 ng/ml OSM (OSM), or growth arrest media with 50 ng/ml IL-6 (IL6).

Figure 5: Growth suppression of MCF-12A cells following C/EBPδ over-expression. MCF-12A cells were transfected with no construct (- Control), full length C/EBPδ construct (Full Length), or pcDNA3 (+ Control) and selected for with G418. Plates were stained with crystal violet and photographed.

Figure 6: Effect of estrogen receptor expression on C/EBPδ induction. **a)** Forty-eight hour [^{3}H]thymidine incorporation of estrogen receptor positive (ER+) and negative (ER-) cells cultured in growth media (GR), growth arrest media (GA), growth arrest media with 50 ng/ml OSM (OSM) or growth arrest media with 50 ng/ml IL-6 (IL6). **b)** [^{3}H]Thymidine incorporation of near confluent plates of MCF-7 cells initially growth arrested in media containing .1% charcoal-stripped serum for 72 hours and then either maintained in growth arrest media (GA-SS) or having 100 pM 17β-estradiol (GA-SS + 100 pM E_2) added for 36 hours. c) [^{3}H]Thymidine incorporation of near confluent plates of MCF-7 cells initially growth arrested in media

containing .1% charcoal-stripped serum for 72 hours and then either maintained in growth arrest media (GA-SS), having 100 pM 17β-estradiol (GA + 100 pM E_2) added, or having 100 pM 17β-estradiol and 100 nM 4-hydroxytamoxifen (GA + 100 pM E_2 + 100 nM 4-OHT) added for 38 hours. **d)** Near confluent plates of MCF-7 cells were maintained in growth arrest media (.1% stripped serum) alone for 72 hours (GA), with the addition of 100 pM E2 (GA + E2), with the addition of 100 pM E2 and 100 nM 4-OHT (GA + E2 + 4OHT), or with the addition of 100 nM 4-OHT (GA + 4-OHT). RNA was isolated and analyzed by Northern blot for C/EBPδ expression. CP was used as a loading control.

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level of ~200-250 adducts/10^8 nucleotides for both PAHs tested and suggest that the level of adducts rather than their structure triggers the p53 and p21^{WAF1} responses. Finally, treatment of growing HEL cells with 0.1 μM DB[a,l]P resulted in major cell cycle alterations starting from 12 to 16 h of treatment. After 24 h of exposure, there was ~24% increase of cells in S phase while the proportions of cells in G0/G1 and G2/mitosis were depressed. Similar trend was observed at near confluence and after exposure to BαP, but differences were more subtle. These data suggest that exposure of HEL cells to DB[a,l]P, and less so to BαP, induces DNA adduct formation, as well as p53 and p21^{WAF1} expression without eliciting transient G1 arrest, but rather an S phase delay/arrest. It is unclear if the increases in both p53 and p21^{WAF1} are responsible for the S phase delay to allow DNA repair before further progression of the cell cycle. Supported by the grant of Ministry of Environment of the Czech Republic VaV/340/2/00.

#3958 Downregulation of the Potential Suppressor Gene IGFBP-rP1 in Human Breast Cancer is Associated with Inactivation of the Retinoblastoma Protein, Cyclin E Overexpression and Increased Proliferation in Estrogen Receptor Negative Tumors. Göran P. Landberg, Hanna Östlund, Angelika M. Burger, and Arun Seth. *Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Canada, Division of Pathology, Malmö, Sweden, and Tumor Biology Center at the University of Freiburg, Freiburg, Germany.*

The complex insulin-like growth factor network of ligands, receptors and binding proteins has been shown to be disturbed in breast cancer. In addition to defects in proteins controlling cell cycle checkpoints, this type of aberrations could affect tumor growth and survival thereby influencing both tumor aggressiveness and potential response to treatments. We have previously identified the T1A12/mac25 protein, which is identical to the IGFBP-rP1, as a differentially expressed gene product in breast cancer cells compared with normal cells. We also found loss of heterozygosity for the IGFBP-rP1 gene during progression to invasive breast cancer suggesting a suppressor-like function for IGFBP-rP1. Here we compare the expression of IGFBP-rP1 in 106 tumor samples with known status of cell cycle aberrations and other clinicopathological data. This was done using a tumor tissue section array system which allows for simultaneous immunohistochemical staining of all samples in parallel. Cytoplasmic staining of variable intensity was observed in most tumors, 15% lacked IGFBP-rP1 staining completely, 20% had weak staining, 32% intermediate and 33% showed strong staining. Low IGFBP-rP1 was associated with high cyclin E protein content, retinoblastoma protein (pRb) inactivation, low bcl-2 protein, poorly differentiated tumors and higher stage. There was a significantly impaired prognosis for patients with low IGFBP-rP1 protein tumors. Interestingly, IGFBP-rP1 showed a strong and inverse association with proliferation (Ki-67 %) in estrogen receptor negative tumors as well as in cyclin E high tumors suggesting a separate cell cycle regulatory function for IGFBP-rP1 independent of interaction with the estrogen receptor pathway and the pRb pathway. MCF-7 and MDA-468 breast cancer cell lines have also been transfected with an IGFBP-rP1 cDNA construct and the IGFBP-rP1 overexpressing cell lines will be used to further investigate the connection between IGFBP-rP1 and proliferation.

#3959 DNA-Dependent Protein Kinase as a Component in the Regulation of DNA Replication Following DNA Damage. Hongyan Wang, Jun Guan, M. Saiful Huq, Ya Wang, and George Iliakis. *Kimmel Cancer Center of Jefferson Medical College, Thomas Jefferson University, Philadelphia, PA.*

Exposure of mammalian cells to DNA damage inducing agents inhibits ongoing DNA replication. It has been documented that a major component of this inhibition derives from a delay in the initiation of unreplicated origins. Although the underlying molecular mechanism of this response remains largely unknown, *ATM* is thought to play an important role. Evidence has been presented that the inhibition observed in DNA replication in response to DNA damage is equivalent to the activation of a checkpoint in S-phase. The molecular determinants of this checkpoint remain to be elucidated. We investigated the regulation of DNA replication after DNA damage using a simian virus 40 (SV40) based *in vitro* DNA replication assay. In this assay, replication of plasmids containing the SV40 origin of DNA (ori$^+$ DNA) replication is accomplished *in vitro* with either crude cytoplasmic extracts, or proteins purified from such extracts with SV40 T antigen (TAg) as the only non-cellular protein. It is believed that all cellular proteins required in this assay function in a manner similar to that *in vivo*. We have reported (Wang et al., J Biol. Chem. 274:22060-22064, 1999) that extracts of cells exposed to DNA damage have reduced activity for *in vitro* DNA replication and inhibit, in a dominant fashion, the ability of extracts from non-treated cells to promote *in vitro* DNA replication. The reduced DNA replication activity in extracts of irradiated cells is partly caused by a reduction in the amount of replication protein A (RPA) and can be corrected by the addition of purified RPA. The dominant component of the inhibition is caused by the activation DNA-dependent protein kinase (DNA-PK), which inactivates SV40 T antigen by phosphorylation. Here we evaluate in detail the parameters that determine the degree of RPA loss from the extracts, and show that the presence of fragmented DNA with single strand overhangs will recruit RPA and compromise DNA replication. The results demonstrate that RPA and DNA-PK are involved in the regulation of viral DNA replication after DNA damage and suggest that analogous processes regulate cellular DNA replication with the DNA-PK targeting the functional homologues of TAg. Since DNA-PK is also intimately involved in DNA DSB repair, a model is developed according to which DNA-PK acts as a local checkpoint to inhibit DNA replication while promoting repair in areas where DNA DSBs have been induced. This work was supported by grants CA76203 (YW), CA56706 (GI), P30-CA56036 and T32-CA09137 (X.Y.Z) from NCI, DHHS.

#3960 Lack of Transforming Growth Factor β Type I Receptor Somatic Mutations in Squamous Cell Carcinomas of the Head and Neck. Thomas J. Knobloch, Huijuan Song, Elizabeth M. Adams, Jas C. Lang, David E. Schuller, and Christopher M. Weghorst. *The Ohio State University, Columbus, OH.*

The transforming growth factor beta receptor (TβR) complex is characterized by the heteromeric association of the transforming growth factor receptor type I (TβR-I) and type II (TβR-II) serine/theonine kinases. Working in obligatory cooperation, TβR-I and TβR-II indirectly regulate the phosphorylation of the retinoblastoma tumor suppressor gene product and subsequent progression of the cell cycle. Inactivation of either TβR-I or TβR-II gene may lead to the deregulation of cell cycle and ultimately uncontrolled proliferation. We have previously reported the identification of mutations within TβR-II from squamous cell carcinomas of the head and neck (SCCHN). In the present study, we analyzed 30 SCCHN and patient matched normal tissues for mutations within TβR-I. The entire coding and flanking intron sequences of TβR-I were screened using "Cold" SSCP and DNA sequencing. No somatic sequence changes were identified. In contrast, 13 of 30 (43%) exhibited polymorphic sequence changes. Seven cases exhibited a 9-bp deletion located within a $(GCC)_9$ microsatellite repeat in exon 1 (5 heterozygous; 2 homozygous), while one case showed a homozygous 12-bp deletion. In addition to these changes within the coding region, one case exhibited a heterozygous C to A substitution at the +24 nucleotide position of intron 5 and five cases demonstrated a heterozygous G to A substitution in intron 7 at position +82. The protein derived from the 9-bp deletion allele has been shown by others to exhibit impaired function. These data suggest that polymorphic substitutions within the TβR-I gene and not somatic mutations, may contribute to SCCHN development via TβR complex inactivation.

#3961 Cloning and Analysis of FKHR Transcription Factor Family Members from a *Xiphophorus Melanoma* Model. Michael D. Rudd and Andrew P. Butler. *University of Texas M.D. Anderson Cancer Center, Smithville, TX.*

AFX, FKHR and FKHR-L1 are vertebrate forkhead transcription factors orthologous to DAF-16, a regulator of longevity in *C. elegans*. Overexpression of any one of the vertebrate factors leads to G1 arrest and/or apoptosis. They are each negatively regulated through the PI3K/AKT signalling pathway, but the immediate downstream target has been identified only for AFX. We are using the *Xiphophorus*fish melanoma model to analyze the contribution of these factors to cell cycle control and development of cancer. *Xiphophorus*hybrids have been widely used to study the genetics of melanoma formation. Towards this end, we have begun cloning FKHR homologs from *Xiphophorus*by performing RT-PCR and 5' / 3' RACE upon total RNA from *X. maculatus*liver. We have isolated 1.7 kb of a cDNA most identical and homologous, at the predicted amino acid level, to the FoxO subclass of forkhead factors, which includes: human FKHR-L1 (53% / 63%; e^{-141}), *Mus musculus*FKHR2 (53% / 63%; e^{-140}) and *Danio rerio*Fish FKHR (54% / 64%; e^{-135}). Notably, the winged helix DNA binding domain and at least two AKT phosphorylation sites are highly conserved. Characterization of *Xiphophorus*-FKHR-related genes will contribute to understanding the molecular basis of carcinogenesis in these species. Supported by CA46629, ES07784 and T32CA09480.

#3962 Increased CCAAT/Enhancer Binding Protein δ (C/EBPδ) Expression in Growth Arrested Human Breast Derived Cell Lines. Gloria S. Sivko and Jim W. DeWille. *The Ohio State University, Dept. Veterinary Biosciences and Comprehensive Cancer Center, Columbus, OH.*

CCAAT/Enhancer binding proteins (C/EBPs) are a highly conserved family of DNA binding proteins. C/EBPs influence cell fate determining pathways (growth, death, differentiation) by functioning as transcription factors and also by interacting with key cell cycle regulatory proteins such as the retinoblastoma protein (Rb). The purpose of this study is to demonstrate the role of C/EBPδ in G_0 growth-arrest of human mammary epithelial cells, both non-tumorigenic and tumor cell lines, and compare it to our previously established mouse model system. Utilizing standard Northern and Western blotting protocols, we demonstrate that C/EBPδ mRNA and protein levels are highly induced in G_0 growth-arrested non-tumorigenic human mammary epithelial cell lines MCF-10A and MCF-12A. In contrast, C/EBPδ mRNA and protein appear to be induced at much lower levels in human breast cancer cell lines (MCF-7, SK-BR-3, T-47D, MDA-MB-231, MDA-MB-468). The relationship between C/EBPδ induction and growth status in these tumor cell lines has been difficult to interpret due to the inability to growth arrest the cells via standard growth factor deprivation methods. Since cytokines of the IL-6 family are known to activate the Jak/STAT pathway and induce growth arrest in some cell types, the effect of IL-6 type cytokine addition (IL-6, Oncostatin M (OSM)) on C/EBPδ induction is currently being investigated in the above cell lines. Preliminary data shows that OSM increases C/EBPδ RNA and protein expression under both growing and growth arrest conditions but is not sufficient to induce growth arrest in growth media. It does appear to promote growth arrest when coupled with growth factor deprivation. These results suggest that C/EBPδ functions in the initiation or maintenance of G_0 growth arrest in mammary epithelial cells, the principal cell population implicated in human breast cancer. This function may be disrupted in human breast cancer cell lines or in clinical breast cancer.

Abstract #30

C/EBPδ EXPRESSION IN HUMAN MAMMARY EPITHELIAL AND PROSTATE CELLS

G. Sivko, D. Sanford and J. DeWille. Dept. of Veterinary Biosciences, MCDB Program, OSUCCC, The Ohio State University, Columbus, OH

The purpose of this study was to investigate the role of C/EBPδ in G_0 growth-arrest of human mammary epithelial cells and prostate cells. C/EBPδ mRNA and protein levels were highly induced in G_0 growth-arrested non-tumorigenic human mammary epithelial cell lines MCF-10A and MCF-12A (G_0 growth arrest was induced by serum and growth factor withdrawal from near (70%) confluent cultures). In contrast, C/EBPδ mRNA and protein appear to be induced at much lower levels in human breast cancer cell lines (MCF-7, SK-BR-3, T-47D, MDA-MB-231, MDA-MB-468) and the PC-3 and LNCaP prostate cell lines. C/EBPδ induction and growth status in human tumor cell lines has been difficult to interpret due to the variability in growth arrest status using standard growth factor deprivation methods. Since cytokines of the IL-6 family activate the Jak/STAT pathway and induce growth arrest in some cell types, the effect of IL-6 type cytokine addition (IL-6, Oncostatin M (OSM)) on C/EBPδ induction was investigated in the above cell lines. Preliminary data in the mammary cell lines shows that the combined effects of serum/growth factor withdrawal and OSM accelerates growth arrest and increases C/EBPδ RNA and protein expression. In human prostate cells (LNCaP), IL-6 treatment rapidly induces C/EBPδ and results in growth arrest, even when added to subconfluent cultures in complete growth media. STAT3 is phosphorylated in all growth arrested and cytokine treated cells. These results demonstrate that C/EBPδ expression is influenced by growth status and cytokine treatment in both the mammary and prostate cell lines. Intracellular signal transduction and promoter regulation studies are currently underway to further understand the cellular mechanisms that control C/EBPδ expression and G_0 growth-arrest in human mammary epithelial and prostate cells.

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THE CCAAT/Enhancer Binding Protein (C/EBP) Gene Promoter is Induced by Activated Signal Transduction and Activator of Transcription3 (STAT3) and Silenced by Hypermethylation

The overall goal of this study was to investigate the regulation of C/EBPδ in immortalized mammary epithelial cells, transformed breast cancer cell lines and primary human breast cancer samples. In previous studies we demonstrated that C/EBPδ functions as a growth suppressor in mouse and human mammary epithelial cells (JBC, 272:6291, 1997, JBC, 275:29123, 2000, Mol. Cancer Therap. 1:610, 2002). In the present study we investigated C/EBPδ gene promoter methylation status in human breast cancer cell lines and primary breast cancer samples. The SUM52PE human breast cancer cell line does not express C/EBPδ. Methylation-specific PCR analysis demonstrated that 26/27 CpG dinucleotides are methylated in the SUM52PE C/EBPδ gene promoter. In contrast, human breast cancer cell lines expressing detectable levels of C/EBPδ exhibited little or no CpG methylation in the C/EBPδ gene promoter. Primary human breast cancer samples that express low levels of C/EBPδ also exhibit C/EBPδ gene promoter methylation in close proximity to consensus transcription factor binding sites. Chromatin immunoprecipitation (ChIP) assays using anti-acetylated H4 antibody demonstrate that the C/EBPδ gene promoter is associated with a closed chromatin conformation in SUM52PE cells. We also investigated the influence of Oncostatin M (OSM) on STAT3 phosphorylation, C/EBPδ gene expression and growth arrest in human and mouse mammary epithelial cells. OSM treatment induced STAT3 phosphorylation, increased C/EBPδ mRNA and protein levels and resulted in growth arrest of mouse and human mammary epithelial cells. Transient transfection assays demonstrate that the consensus STAT3 site in both the mouse and human C/EBPδ gene promoters is essential for OSM-mediated activation of the C/EBPδ promoter. Co-transfection with dominant negative STAT3 blocks OSM activation of the C/EBPδ promoter-luciferase constructs. These results demonstrate that: (1) The C/EBPδ gene is silenced by extensive promoter methylation in SUM52PE cells. Site-specific CpG methylation may alter transcription factor binding sites on the C/EBPδ gene promoter in primary breast tumors. (2) OSM induces STAT3 phosphorylation, increases C/EBPδ gene promoter activation and results in growth arrest of mammary epithelial cells. These results indicate that silencing of the C/EBPδ gene by methylation may contribute to defective growth control in human breast cancer. OSM activation of STAT3 may provide a potential therapeutic approach to activate C/EBPδ gene expression and induce growth arrest in breast cancer cells cancer cells. This work was supported by NCI, CA57607.

Primary Program Affiliation: RNA Oncogenic Virus

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C/EBP-DELTA: A POTENTIAL BREAST CANCER TUMOR SUPPRESSOR GENE

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The overall goal of this research is to investigate the role of C/EBP-delta in human mammary epithelial cell growth control. We previously demonstrated that C/EBP-delta functions as a growth suppressor in mouse mammary epithelial cells (JBC, 272:6291, 1997, JBC, 275:29123, 2000). In the present study, we show that serum and growth factor withdrawal, or Oncostatin M (OM) treatment induces Stat3 activation (phosphorylation), increases C/EBP-delta gene expression and results in G0 growth arrest of primary and immortalized (MCF-10A, MCF12A) human mammary epithelial cells. In contrast, serum and growth factor withdrawal and OM treatment is associated with variable Stat3 activation, reduced C/EBP-delta gene expression and aberrant G0 growth arrest in human breast cancer cell lines (MCF-7, MDA-MB-231, SK-BR-3, T47-D, SUM-44PE, SUM-52PE and SUM-102PT). C/EBP-delta gene structure and promoter methylation status were investigated in human breast cancer cell lines by single strand conformation polymorphism (SSCP) analysis, direct genomic sequencing and bisulfite /methylation-specific PCR. The sequencing results identified a nonsense mutation (A538T, AAG -> TAG) in the SUM-52PE C/EBP-delta gene. In addition, bisulfite/methylation-specific PCR analysis demonstrated that 25/27 CpGs were methylated in the SUM-52PE C/EBP-delta gene promoter. Limited CpG methylation was also observed in the T47-D C/EBP-delta gene promoter. The results from this study demonstrate that: (1) C/EBP-delta functions in the regulation of normal human mammary epithelial cell G0 growth arrest. This growth control function is compromised in human breast cancer cell lines. (2) STAT3 activation (phosphorylation) is associated with the induction of C/EBP-delta gene expression in G0 growth arrested human mammary epithelial cells. (3) A nonsense mutation (AAG -> TAG) is present in the SUM-52PE C/EBP-delta coding region. (4) The SUM-52PE C/EBP-delta gene promoter is heavily methylated (25/27 CpGs). Extensive methylation of the C/EBP-delta gene promoter is significant as the SUM-52PE cell line does not express the C/EBP-delta gene. The results of the present study support a tumor suppressor role for C/EBP-delta in human breast cancer.

The U.S. Army Medical Research and Materiel Command under DAMD17-00-1-0389 supported this work.

variant of mutant p53 (mut aa 175) is able to disrupt ER binding at these sites. In addition, overexpression of this dominant-negative p53 variant is able to inhibit the estrogen-mediated transcription from pGL3 BRCA-1 in ER+ cells. Taken together, these data suggest that regulation of BRCA-1 expression by estrogen may require functional ERα and p53 pathways.

#4230 The activated aromatic hydrocarbon receptor regulates BRCA-1 promoter activity at xenobiotic responsive elements. Chi-Fan Ku, Brandon D. Jeffy, and Donato F. Romagnolo. *University of Arizona, Tucson, AZ.*

In the absence of a causal relationship between the incidence of mutations in the breast cancer susceptibility BRCA-1 gene and occurrence of sporadic breast cancer, investigations of whether endogenous factors contribute to the etiology of mammary tumors induced by environmental xenobiotics are warranted. Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous pollutants, which have been shown to induce mammary tumors in rodents, DNA damage, and disrupt cell cycle progression. Previous work in our laboratory (Cancer Res.62:113) documented that benzo[a]pyrene (B[a]P), a prototype PAH, repressed BRCA-1 promoter activity in breast and ovarian estrogen receptor positive (ER+) cells. In this study, we investigated whether disruption of BRCA-1 transcription by B[a]P was mediated by the aryl hydrocarbon receptor (AhR) and the AhR-nuclear transporter (ARNT) protein at xenobiotic responsive elements (XRE) flanked in the 5' flanking region of the BRCA-1 gene. Analysis of the BRCA-1 gene indicated that several XREs are strategically located at -539 base pairs (bp) (CCGTGGAA=Cyp1A1-like) and +20 bp (GCGTG=XRE-1) from the transcription start site on exon-1A. Two additional XREs (GCGTG) have been localized at -107 bp in the intervening sequence upstream (XRE-2) and +218 bp (XRE-3) into exon-1B. Promoter deletion and site-directed mutagenesis studies of the candidate XREs documented that the CYP1A1-like element may be required for constitutive activity of the BRCA-1 promoter. Conversely, the XRE-1 and XRE-2 may function as negative regulators of BRCA-1 transcription. Electromobility binding and shift assay experiments confirmed that the candidate BRCA-1 XREs are targets by nuclear protein complexes containing the AhR. These findings suggest that the activated AhR may bind to XREs harbored in the flanking region of the BRCA-1 gene, thus regulating its expression. A potential implication of these observations is that exposure to PAHs may be a risk factor in the etiology of sporadic breast cancer.

#4235 The CCAAT/Enhancer Binding Proteinδ (C/EBPδ) gene promoter is induced by activated signal transduction and activator of transcription3 (STAT3) and silenced by hypermethylation. Dahai Tang, Gloria S. Sivko, Yingjie Zhang, and James DeWille. *Ohio State University, Columbus, OH.*

The overall goal of this study was to investigate the regulation of C/EBPδ in immortalized mammary epithelial cells, transformed breast cancer cell lines and primary human breast cancer samples. In previous studies we demonstrated that C/EBPδ functions as a growth suppressor in mouse and human mammary epithelial cells (JBC, 272:6291, 1997, JBC, 275:29123, 2000, Mol. Cancer Therap. 1:610, 2002). In the present study we investigated C/EBPδ gene promoter methylation status in human breast cancer cell lines and primary breast cancer samples. The SUM52PE human breast cancer cell line does not express C/EBPδ. Methylation-specific PCR analysis demonstrated that 26/27 CpG dinucleotides are methylated in the SUM52PE C/EBPδ gene promoter. In contrast, human breast cancer cell lines expressing detectable levels of C/EBPδ exhibited little or no CpG methylation in the C/EBPδ gene promoter. Primary human breast cancer samples that express low levels of C/EBPδ also exhibit C/EBPδ gene promoter methylation in close proximity to consensus transcription factor binding sites. Chromatin immunoprecipitation (ChIP) assays using anti-acetylated H4 antibody demonstrate that the C/EBPδ gene promoter is associated with a closed chromatin conformation in SUM52PE cells. We also investigated the influence of Oncostatin M (OSM) on STAT3 phosphorylation, C/EBPδ gene expression and growth arrest in human and mouse mammary epithelial cells. OSM treatment induced STAT3 phosphorylation, increased C/EBPδ mRNA and protein levels and resulted in growth arrest of mouse and human mammary epithelial cells. Transient transfection assays demonstrate that the consensus STAT3 site in both the mouse and human C/EBPδ gene promoters is essential for OSM-mediated activation of the C/EBPδ promoter. Co-transfection with dominant negative STAT3 blocks OSM activation of the C/EBPδ promoter-luciferase constructs. These results demonstrate that: (1) The C/EBPδ gene is silenced by extensive promoter methylation in SUM52PE cells. Site-specific CpG methylation may alter transcription factor binding sites on the C/EBPδ gene promoter in primary breast tumors. (2) OSM induces STAT3 phosphorylation, increases C/EBPδ gene promoter activation and results in growth arrest of mammary epithelial cells. These results indicate that silencing of the C/EBPδ gene by methylation may contribute to defective growth control in human breast cancer. OSM activation of STAT3 may provide a potential therapeutic approach to activate C/EBPδ gene expression and induce growth arrest in breast cancer cells. This work was supported by NCI, CA57607.

#4236 Abnormalities of hepatocyte nuclear factor 3 beta in lung cancer. Balazs Halmos, Daniela Basseres, Tajhal Dayaram, Katalin Ferenczi, Claudia S. Huettner, and Daniel G. Tenen. *Beth Israel Deaconess Medical Center, Boston, MA and Brigham and Women's Hospital, Boston, MA.*

Previously we have shown that hepatocyte nuclear factor 3 beta (HNF3beta) is a potential target gene of C/EBPalpha, a differentiation gene and candidate tumor suppressor in airway epithelium (Halmos et al, AACR 2002). HNF3beta (also called Foxa2) is a member of the forkhead transcription factor family, it is involved in airway differentiation and regulates the transcription of a number of important lung-specific genes. We have determined the expression of HNF3beta in 25 lung cancer cell lines by Northern blotting and found that, while HNF3beta expression was strong in normal lung, it was either undetectable or very weak in 15/25 cancer cell lines examined. Western blotting analysis showed strong correlation between mRNA and protein levels in 20/22 cell lines examined. In addition, aberrant mRNA specimens were observed in some cell lines. As part of our study to determine the identity of these aberrant transcripts and to determine the presence of other molecular alterations of the HNF3beta gene in lung cancer, we performed genomic sequencing of part of the promoter and all 3 exons of the HNF3B gene in 32 lung cancer cell lines and found mutations in 2 cell lines. One cell line, H60 (small cell lung cancer) has a heterozygous G-A mutation at position 2916 (GenBank accession AF176110) resulting in a G-D amino acid change at codon 92 inside the N-terminal activation domain. A homozygous C deletion at codon 194 (position 3220 in AF176110) in the middle of the forkhead domain was found in the SKLU-1 cell line (adenocarcinoma) leading to a frameshift and a truncated 218 amino acid protein. The truncated form was not detected in Western blots and the mRNA is not expressed either which raises the possibility that this mutation might alter the stability of the mRNA. To our knowledge these are the first mutant forms of HNF3beta ever described. The functional significance of these mutations is being assessed. We are currently performing sequencing on laser microdissected tumor specimens to confirm the presence of such mutants in primary specimens. In addition, four cell lines had silent basepair changes probably representing single nucleotide polymorphisms in the coding region, 2 cell lines had a G-A basepair change at position -17 in the promoter and one cell line had an A-G nucleotide change in the 3' UTR. In order to further analyze the role of HNF3beta in airway epithelium, we used a tetracycline-inducible system to express HNF3beta in the H358 cell line (in which we originally described HNF3beta inducibility by C/EBPalpha). The induction of a 3-fold increase in HNF3beta protein led to a 60% growth reduction within 7 days. Further characterization of the cellular changes, including apoptosis, proliferation and differentiation as a result of HNF3beta expression are ongoing. In summary, our results suggest widespread abnormalities of HNF3beta in lung cancer as well as demonstrate the growth-inhibitory role of HNF3beta in airway epithelial cells.

#4237 Linking proliferation and the block to differentiation in leukemia: A potential regulatory loop between CDK6 and PU.1. Kevin S. Choe, Igor Matushansky, Natasha Rekhtman, and Arthur I. Skoultchi. *Department of Cell Biology, Albert Einstein College of Medicine, Bronx, NY.*

Characteristic features of leukemias and many other cancers include both uncontrolled proliferation and a block to terminal differentiation.

CCAAT/ENHANCER BINDING PROTEIN δ (C/EBPδ): A CANDIDATE BREAST CANCER TUMOR SUPPRESSOR GENE. G. Sivko and J. DeWille. Department of Veterinary Biosciences, College of Veterinary Medicine, OSU.

CCAAT/Enhancer binding proteins (C/EBPs) are a highly conserved family of DNA binding proteins. C/EBPs influence cell fate determining pathways (growth, death, differentiation) by functioning as transcription factors and also by interacting with key cell cycle regulatory proteins such as the retinoblastoma protein (Rb). We recently showed that C/EBPδ gene expression is increased (along with p27) in G_0 growth arrested mouse mammary epithelial cells (J. Biol. Chem., 274:16582, 1999) and is regulated by the Jak/STAT signal transduction pathway (J. Biol. Chem., in press). We also showed that reducing mammary epithelial cell C/EBPδ levels by antisense expression delayed G_0 growth arrest and apoptosis. In contrast, increasing C/EBPδ levels by constitutive overexpression accelerated G_0 growth arrest and apoptosis. C/EBPδ is also induced during Stage I (early apoptosis) in the involuting mouse mammary gland (Breast Canc. Res. & Trt. 58:57, 1999). Most (80%) of mouse mammary tumors derived from MMTV/c-neu transgenic mice express low levels of C/EBPδ mRNA.

In this study we demonstrate that C/EBPδ mRNA and protein levels are induced in G_0 growth-arrested non-tumorigenic human mammary epithelial cell lines MCF-10A and MCF-12A. In contrast, C/EBPδ mRNA and protein appear to be induced at lower levels in human breast cancer cell lines (MCF-7, SK-BR-3, T-47D, HT-1080, MDA-MB-231, MDA-MB-468). The relationship between C/EBPδ induction and growth status in these cell lines has been difficult to interpret due to the inability to growth arrest the cells via standard growth factor deprivation methods. Since cytokines of the IL-6 family are known to activate the Jak/STAT pathway and induce growth arrest in some cell types, the effect of IL-6 type cytokine addition (IL-6, OncostatinM) on C/EBPδ induction is currently being investigated in the above cell lines. Preliminary data shows that OncostatinM (OSM) increases C/EBPδ RNA and protein expression under both growing and growth arrest conditions but is not sufficient to induce growth arrest in growth media. It does appear to promote growth arrest when coupled with growth factor deprivation. These results suggest that C/EBPδ functions in the initiation or maintenance of G_0 growth arrest in mammary epithelial cells, the principal cell population implicated in human breast cancer. This function may be disrupted in human breast cancer cell lines, or in clinical breast cancer. Few G_0 genes have been identified and little is known about the control of cell cycle exit/G_0 entry, or the regulation and function of genes that are expressed during G_0 growth arrest. We are currently investigating the regulation of C/EBPδ gene expression during G_0 growth arrest, as well as the structure and function of C/EBPδ gene in normal and breast cancer samples.

Abstract #4235

The CCAAT/Enhancer Binding Proteinδ (C/EBPδ) Gene Promoter is Induced by Activated Signal Transduction and Activator of Transcription3 (STAT3) and Silenced by Hypermethylation.

Danai Tang, Gloria S. Sivko, Yingjie Zhang, James DeWille, Ohio State University, Columbus, OH.

Abstract

The overall goal of this study was to investigate the regulation of C/EBPδ in nontransformed mammary epithelial cells, transformed breast cancer cell lines and primary human breast cancer samples. In previous studies we demonstrated that C/EBPδ functions as a growth suppressor in mouse and human mammary epithelial cells (JBC, 272:6291, 1997, JBC, 275:20129, 2000, Mol. Cancer Therap. 1:619, 2002). In the present study we investigated C/EBPδ gene promoter methylation status in human breast cancer cell lines and primary breast cancer samples. The SUM52PE human breast cancer cell line does not express C/EBPδ. Methylation-specific PCR analysis demonstrated that 26/27 CpG dinucleotides are methylated in the SUM52PE C/EBPδ gene promoter. In contrast, human breast cancer cell lines expressing detectable levels of C/EBPδ exhibited little or no CpG methylation in the C/EBPδ gene promoter. Primary human breast cancer samples that express low levels of C/EBPδ also exhibit C/EBPδ gene promoter methylation in close proximity to consensus transcription factor binding sites. Chromatin immunoprecipitation (ChIP) assays using anti-acetylated H4 antibody demonstrate that the C/EBPδ gene promoter is associated with a closed chromatin conformation in SUM52PE cells. We also investigated the influence of Oncostatin M (OSM) on STAT3 phosphorylation, C/EBPδ gene expression and growth arrest in human and mouse mammary epithelial cells. OSM treatment induced STAT3 phosphorylation, increased C/EBPδ mRNA and protein levels and resulted in growth arrest of mouse and human mammary epithelial cells. Transient transfection assays demonstrate that the consensus STAT3 site in both the mouse and human C/EBPδ gene promoters is essential for OSM-mediated activation of the C/EBPδ promoter. Co-transfection with dominant negative STAT3 blocks OSM activation of the C/EBPδ promoter-luciferase constructs. These results demonstrate that: (1) The C/EBPδ gene is silenced by extensive promoter methylation in SUM52PE cells. Site-specific CpG methylation may alter transcription factor binding sites on the C/EBPδ gene promoter in primary breast tumors. (2) OSM induces STAT3 phosphorylation, increases C/EBPδ gene promoter activation and results in growth arrest of mammary epithelial cells. These results indicate that silencing of the C/EBPδ gene by methylation may contribute to defective growth control in human breast cancer. OSM activation of STAT3 may provide a potential therapeutic approach to activate C/EBPδ gene expression and induce growth arrest in breast cancer cells cancer cells. This work was supported by NCI, CA57607.

Introduction

- Breast cancer is the second leading cause of cancer-related deaths in US women with 180,000 new cases of breast cancer diagnosed each year in the US.
- "Loss of function" changes in growth control pathways play a role in the development and progression of breast cancer.
- C/EBPδ is a leucine zipper transcription factor which functions as a growth suppressor in mouse and human mammary epithelial cells.
- C/EBPδ expression is induced by activation of the Jak/Stat3 signaling pathway following serum and growth factor withdrawal.
- IL-6 family cytokines (IL-6, OSM) have been shown to activate the Jak/Stat signaling pathway.
- C/EBPδ expression is decreased in breast cancer cell lines and primary breast tumors.
- Promoter methylation is one form of gene inactivation exhibited by cancer cells.
- **Hypothesis:** C/EBPδ expression is regulated by Stat3 binding to the C/EBPδ promoter following serum and growth factor withdrawal and Oncostatin M (OSM) addition. Decreased C/EBPδ expression in breast cancer cell lines and primary breast tumors is due to methylation of CpG islands in the C/EBPδ promoter.

Methods and Results

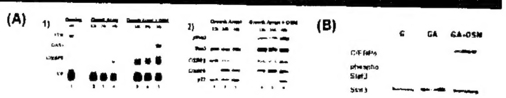

FIGURE 1: The Effect of OSM on Stat3 Phosphorylation, C/EBPδ mRNA and Protein Expression and G_0 Growth Arrest of Mouse and Human Mammary Epithelial Cells.

Conclusion: Serum and growth factor withdrawal (GA) or OSM addition induces Stat3 phosphorylation (activation), increases C/EBPδ mRNA and protein levels and results in G_0 growth arrest of human MCF-12A and mouse HC11 cells.

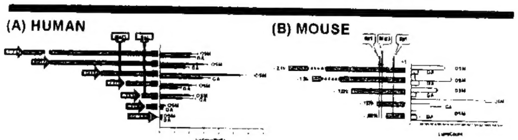

FIGURE 2: Mapping the Stat3 regulated region in the human and mouse C/EBPδ promoter.

Conclusion: Both the human and mouse C/EBPδ gene promoters are activated by GA and OSM in mammary epithelial cells. OSM-specific induction of C/EBPδ promoter activity is located within the -233 bp to -393 bp region of the human C/EBPδ promoter and within the -81 bp to -127 bp region of the mouse C/EBPδ promoter. These OSM-inducible C/EBPδ gene promoter regions contain Stat3 binding sites: -274 to -282 bp in the human; -102 to -109 bp in the mouse. These results demonstrate that activation of the C/EBPδ promoter in both the human and mouse requires Stat3 binding. In both the human and mouse C/EBPδ promoter, removal of a downstream Sp1 site also diminishes promoter activity.

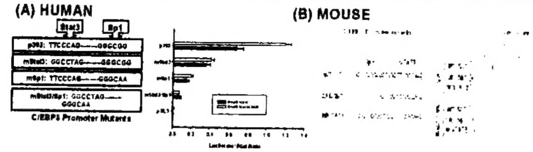

Figure 3: Activation of the human and mouse C/EBPδ promoters requires both the Stat3 and Sp1 binding sites.

Conclusion: Mutation of the Stat3 site in the human promoter decreases C/EBPδ promoter activity by over 50% under both growth arrest and OSM treated conditions. Mutation of the downstream Sp1 site and mutation of both the Stat3 and downstream Sp1 sites decreases C/EBPδ promoter activity to almost basal levels. In the mouse promoter, deletion of the Sp1 site upstream of the Stat3 site, or mutation of the Stat3 site significantly reduces C/EBPδ promoter activity in both growth arrest and OSM treated conditions. These results indicate that both activated Stat3 and Sp1 are key transcriptional regulators in the induction of the C/EBPδ gene promoter. The location of the required Sp1 site, however, varies between the two species.

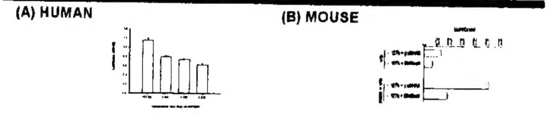

Figure 4: Activation of the human and mouse C/EBPδ promoters is decreased by the addition of dominant negative Stat3.

(A) HUMAN: Luciferase activities following the incremental cotransfection of 2, 4, and 8 µg dominant negative Stat3β (graciously provided by Dr. Richard Jove) with 1 µg -393 bp promoter construct under growth arrest conditions. Total transfected DNA was held constant by the addition of pSG5.
(B) MOUSE: Luciferase reporter assays from -127 bp promoter construct cotransfected with dominant negative Stat3 expression vector under GA and GA+OSM conditions.

Conclusion: Dominant negative Stat3 significantly reduces both human and mouse C/EBPδ promoter activity under growth arrest or growth arrest + OSM conditions confirming the role of activated Stat3 in C/EBPδ promoter induction.

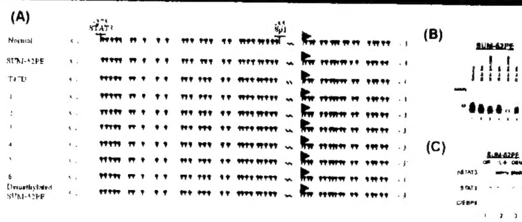

Figure 5: Methylation of the C/EBPδ promoter decreases C/EBPδ expression in breast cancer cell lines and primary tumors.

(A) Sequencing results of MSP products demonstrate that CpG dinucleotides in the C/EBPδ promoter of normal human DNA are unmethylated, however, CpG dinucleotides in the C/EBPδ promoter of the SUM-52PE human breast cancer cell line are heavily methylated (26/27). Limited methylation is detected in CpG dinucleotides in the C/EBPδ gene promoter of primary breast tumor specimens and breast tumor cell lines.
(B) Northern blot indicates that the SUM-52PE cell line does not express detectable levels of C/EBPδ mRNA (CP - loading control).
(C) Western blot of SUM-52PE probed with antibodies to phospho-Stat3(Tyr705), Stat3, and C/EBPδ.

Conclusion: Methylation of the C/EBPδ promoter in breast cancer cell lines and primary breast tumors decreases C/EBPδ transcription. This results in decreased mRNA and protein expression despite the presence of intact upstream signaling pathways.

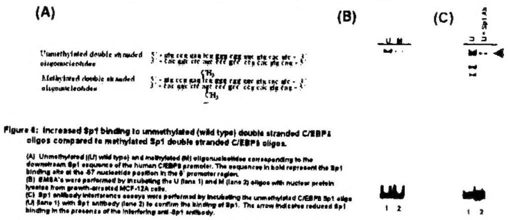

Figure 6: Increased Sp1 binding to unmethylated (wild type) double stranded C/EBPδ oligos compared to methylated Sp1 double stranded C/EBPδ oligos.

Conclusion: Sp1 binding is influenced by the presence of methylated CpGs in close proximity to the consensus Sp1 binding site in the human C/EBPδ gene promoter. Reduced Sp1 binding may play a role in the reduced C/EBPδ gene expression observed in some primary human breast cancer patients.

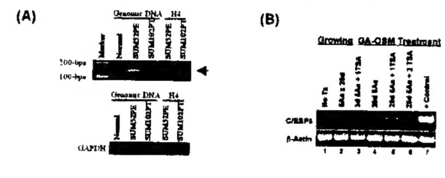

Figure 7: Chromatin precipitation (ChIP) assay demonstrates that C/EBPδ promoter CpG methylation is associated with altered chromatin structure and decreased C/EBPδ expression in the SUM-52PE human breast cancer cell line.

(A) ChIP assays were performed using an anti-acetylated Histone H4 antibody, followed by PCR amplification with human C/EBPδ promoter specific primers. No PCR products were amplified from SUM-52PE ChIP DNA, however, PCR amplified products were obtained from ChIP DNA derived from SUM-102PT human breast cancer cell line (a constitutively high C/EBPδ expressing cell line). PCR products from the GAPDH promoter region were used as a positive ChIP assay control.
(B) SUM-52PE cells were cultured in 30µM 5-Azacytidine for 20 days. Cells were then divided into two groups, growing and growth arrest plus 50 ng/ml OSM. Cells were growth arrested for 3 days in 5-Azacytidine with and without the addition of trichostatin A. Total RNA was isolated, DNase treated and reverse transcribed, followed by PCR with C/EBPδ coding region specific primers. MCF-12A growth arrest mRNA was used as a positive control.

Conclusion: The SUM-52PE human breast cancer cell line does not express C/EBPδ due to dense methylation (26/27 CpGs are methylated) of the C/EBPδ promoter. The SUM-52PE cells exhibit a closed chromatin structure which is reversed with prolonged 5-Azacytidine treatment and Trichostatin A addition.

Conclusions

- Serum and growth factor withdrawal and OSM addition activates Stat3, increases C/EBPδ mRNA and protein expression and results in the G_0 growth arrest of mouse and human mammary epithelial cells.
- Functional Stat3 and Sp1 binding sites in the mouse and human C/EBPδ promoters are required for maximal promoter activation.
- C/EBPδ promoter activity is decreased in breast cancer cell lines and primary breast tumors. This is potentially due to CpG methylation in the Stat3 and Sp1 binding site regions of the C/EBPδ promoter.
- ChIP assays demonstrate a closed chromatin structure associated with increased methylation of the C/EBPδ promoter in the SUM-52PE breast cancer cell line. Treatment with demethylating agents and histone deacetylase inhibitors restores C/EBPδ gene expression.

Acknowledgements

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